BACTERIAL DISEASES AND THEIR MANAGEMENT IN CHOSEN MARINE ORNAMENTAL FISHES

THESIS SUBMITTED
IN PARTIAL FULFILMENT OF THE REQUIREMENTS
FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

IN
MARICULTURE

OF THE
CENTRAL INSTITUTE OF FISHERIES EDUCATION
(DEEMED UNIVERSITY)
VERSOVA, MUMBAI – 400 061

BY

PRAMILA S.
(Ph. D. 57)

भाकृअप
ICAR

CENTRAL MARINE FISHERIES RESEARCH INSTITUTE
(INDIAN COUNCIL OF AGRICULTURAL RESEARCH)
P. B. NO. 1603, KOCHI – 682 014
INDIA

FEBRUARY 2002

भा कृ अनु प
I C A R

Phone: (Off) : 394867/.....Ext 391407
Telegram : CADALMIN EKM
Telex : 0885-6435 MFRI IN
Fax : 91-484-394909
E-mail : mdcmfri@md2.vsnl.net.in

केन्द्रीय समुद्री मात्स्यिकी अनुसंधान संस्थान
(भारतीय कृषि अनुसंधान परिषद)
पोस्ट बॉक्स सं 1603, एरणाकुलम, कोचीन-682 014

CENTRAL MARINE FISHERIES RESEARCH INSTITUTE
(Indian Council of Agricultural Research)
POST BOX No. 1603, ERNAKULAM, COCHIN-682 014

Dated: 25 February 2002

CERTIFICATE

Certified that the thesis entitled **"BACTERIAL DISEASES AND THEIR MANAGEMENT IN CHOSEN MARINE ORNAMENTAL FISHES"** is a record of independent bonafide research work carried out by **Ms. Pramila S.** during the period of study from September 1998 to August 2001 under our supervision and guidance for the degree of **Doctor of Philosophy (Mariculture)** and that the thesis has not previously formed the basis for the award of any degree, diploma, associateship, fellowship or any other similar title.

Major Advisor/Chairman

(A. P. Lipton)
Principal Scientist
VRC of CMFRI

Advisory Committee

(G. Gopakumar)
Principal Scientist
VRC of CMFRI

Rani Mary George
(Rani Mary George)
Principal Scientist
VRC of CMFRI

(G. P. Kumaraswami Achari)
Principal Scientist (Retd.)
VRC of CMFRI

(M. Srinath)
Principal Scientist and Head, FRAD
CMFRI, KOCHI

DECLARATION

I hereby declare that the thesis entitled **"BACTERIAL DISEASES AND THEIR MANAGEMENT IN CHOSEN MARINE ORNAMENTAL FISHES"** is an authentic record of work done by me and that no part thereof has been presented for the award of any degree, diploma, associateship, fellowship or any other similar title.

(Pramila S.)
Ph. D. Scholar,
Post Graduate Programme in Mariculture,
C.M.F.R.I.

Cochin
28-02-02

ACKNOWLEDGEMENTS

With deep gratitude and respect I place on record, heartfelt thanks to ***DR. A. P. Lipton****, Principal Scientist, Chairman and Major Advisor, who took interest in the present area of study and helped me make the results achievable in the face of all hardships and suggested possible solutions wherever hindrances were encountered. His valuable guidance, practical approach to all the problems, and willingness to offer support at all times is thankfully acknowledged.*

I wish to express sincere thanks to ***DR G. Gopakumar****, Principal Scientist, Officer in Charge, Marine Aquarium, VRC of CMFRI for helping me with valuable hints on collection, maintenance and biology of marine ornamental fish, and for the encouragement offered right through the period of study as the Co-Chairman of Advisory committee.*

With gratefulness I remember the spiritual support and timely advice of ***DR. Rani Mary George****, Principal Scientist and Member of Advisory committee. I wish to record my appreciation of* ***Shri. G. P. Kumaraswami Achari****, Principal Scientist (Retd) and Member of Advisory committee for the attention shown in the field of study and for suggestions. I also owe sincere thanks to* ***DR. M. Srinath****, Principal Scientist, Head, FRAD and Member of Advisory committee, who helped me with the statistical analysis part.*

I thank ***DR. M. Devaraj*** *and* ***DR. V. N. Pillai****, former directors of CMFRI and* ***DR. Mohan Joseph Modayil****, present director, for providing me with facilities and the required assistance in Vizhinjam R. C and in Mandapam Regional Centre, which were the places where the work was carried out. The facilities provided by* ***Shri. K. Prabhakaran Nair,*** *former Officer in Charge, VRC and* ***DR. P. P. Pillai****, present OIC are gratefully acknowledged. I take this opportunity to thank* ***DR. N. Kaliaperumal****, Officer in Charge, MRC for making available all facilities required for work in the centre.*

I am very much obliged to ***DR. R. Paul Raj****, Officer in Charge, PGPM for the invaluable support he had endowed me with, the encouragement he had given, as well as for his thoughtfulness, all the way through my tenure in CMFRI.*

I am also deeply indebted to ***Dr. P. C. Thomas****, Principal Scientist, PNPD, and* ***Shri. M. P. Paulton****, Technical Staff, PNPD who helped me execute the electrophoresis studies. Thanks are also due to* ***DR. Razool****, Scientist, Rajiv Gandhi Centre for Biotechnology, Trivandrum,* ***Shri. Bobby Ignatius****, Scientist, MRC,* ***Dr. Joseph Kattikkaran****, Medical Trust Hospital, and* ***DR V. Chandrika****, Principal Scientist, FEMD for the backing offered by all of them.*

I would also like to thank ***DR. C. Suseelan****, former Officer in Charge, PGPM for granting the facilities required for work. I am thankful to all the technical and non-technical staff of PGPM and Establishment section for their assistance in all the official procedures connected with research work.*

The assistance of Shri.Shashi, former staff of VRC, Shri. Seeni and Shri. Ganesan of MRC, Shri. Udayakumar, Shri. Benziger and Shri. Unnikrishnan is particularly acknowledged. The efforts of all the contract labourers who have been of help especially Nazeem, Sivankutty and Senthilkumar are also thanked.

Special thanks also go to my collegues in VRC and MRC- Selvin, Anitha Rani, Anitha, Sabu, batchmates- Sherly, Soni, Suja, Sunil, seniors- Ranjit and Sandhya, for their concern and timely help and all other friends in Trivandrum, Mandapam and Kochi for their encouragement.

The senior research fellowship offered by CIFE is also thankfully acknowledged.

Last but not the least, I avail this opportunity to appreciate the care and affection of my parents and sister. The prayers and love of my beloved friends-Rachel, Munnu, Suby, Princy, Nisha and Seema, are also heartily acknowledged. Without these people this assignment would have been really difficult.

सारांश

केंद्रीय समुद्री मात्स्यिकी अनुसंधान संस्थान के विषिंजम अनुसंधान केंद्र की समुद्री जलजीवशाला में समुद्री आलंकारिक मछलियों में रोग का आकलन और पारिस्थितिक प्राचलों का मानीटरिंग किया गया. यह नोट कर लिया गया कि जलजीवशाला में सूक्ष्म जीवों की अधिक मात्रा जलराशिकी के किसी एक प्रचाल पर प्रभावित नहीं हो जाता है. लेकिन जलजीवशाला में पालित मछली पर विभिन्न प्राचलों का मिश्रित असर ज़रूर पडता है. मुख्य रूप से जल तथा वायुमंडलीय तापमान, नाइट्राइट, विलीन ओक्सिजन व पी एच जैसे घटकों का विश्लेषण करने पर देखा गया कि इन तीनों घटकों द्वारा पानी की गुणता में परिवर्तन हो जाता है. यह अध्ययन सूचित करता है कि सभी मछली वर्गों में जलजीवशाला पुनः सज्जित करने पर होने वाले नाइट्रेट और नइट्राइट के प्राचलों का असंतुलन भारी मृत्युता का कारण बन जाता है. जलजीवशाला मछलियों के बैक्टीरिया विलगनों को फ्लेवोबैक्टीरियम (27%) , विब्रियो (18%) स्यूडोमोनास (16%) आल्कलिजेन्स (16%) , असिनेटोबाक्टर (11%) और एन्टरोबैक्टीरिएसिए कुडुम्ब के सदस्य (8%) के रूप में पहचाना गया है. मछलियों में व्रण-धाव रोग के कारक बैक्टीरिया *सेरेशिया मर्सेसेन्स* का पहली बार विलगन किया गया. यह जाति अत्यंत प्रोटीनलयी स्वभाव की होती है और प्राथमिक अध्ययन के परिणामों के आधार पर इसे परीक्षण जीव यानी क्लाउन मछली (आम्फीप्रियोन सेबे ब्लीकर) में प्रेरित रोगजनता में इसके स्थान पर अनुसंधान करने के लिए चुना गया. मृत्युता के लिए कारक जीवंत कोशों की सांद्रता प्रति ग्राम मछली केलिए $1x10^5$ कोश का समतुल्य देखा गया. *सेरेशिया मर्सेसेन्स* का कोशिकाबाह्य उत्पाद (ई सी पी) परीक्षण जीव केलिए अत्यंत हानिकारक है. ई सी पी की संक्रामक सांद्रता प्रति मछली के शरीर भार का 2.67 मइक्रो ग्राम आकलित किया गया. ई सी पी प्रोटीनों का ताप-आस्थिर स्वभाव प्रयोगशाला अध्ययनों में आकलित किया गया. 10 मिनट तक $50^{\circ}c$ का ऊष्मा उपचार करने पर जेलाटिनेस और केसीनेस की सक्रियता कम हो गया. एस डी एस-पेज अध्ययनों के आधार पर आकलित किया गया कि रोगजनकता के कारक मुख्य प्रोटीन 11 kDa से 100 kDa के रेंच का अणु भार होने वाले हैं और इन में से अधिकांश प्रोटीन 40 kDa से ज्यादा अणु भार वाले हैं. प्रमुख बैक्टीरिया विलगनों की प्रतिजैविकी सुग्राह्यता (एन्टिबयोटिक ससेप्टिबिलिटी) ने यह दिखाया कि परीक्षित 90% से ज्यादा विलगनों में जेन्टामाइसिन, स्ट्रेप्टोमाइसिन , टेट्रासाइक्लिन , नालिडिक्सिक आसिड और साइप्रोफ्लोक्सासिन बढती संदमन करने लायक हैं. *सेरेशिया मर्सेसेन्स* द्वारा होने वाला संक्रामण नियंत्रित करने के लिए प्रभावकारी प्राकृतिक संघटकों, एन्टिसेप्टिक और ऑटोजीनस वाक्सिनों पर परीक्षण किया गया. स्पंजों (*सिग्मोडोसिया कार्नोसा , कालिस्पंजिया* और एक पहचान नहीं की गई जाति) और दो समुद्री शैवालों के सार अच्छे संदमन स्वाभाव के देखे गए. एन्टिसेप्टिकों में फोर्मलीन, एक्रिफ्लविन और मालाकाइट ग्रीन अत्यंत प्रभावकारी देखे गए. बैक्टरिन के प्रयोग के 35 वां और 50 वां दिन के बाद यह साबित किया जा सका कि *सेरेशिया मर्सेसेन्स* बाक्टरिन उपयुक्त करके 100% पेर्सन्टेज रिलेटीव प्रोटक्शन (पी आर पी) प्राप्त कर सकता है.

ABSTRACT

Observations on the disease occurrence among marine ornamental fish and monitoring of environmental parameters were carried out in the marine aquarium, Vizhinjam Research Centre of C. M. F. R. I. It was noted that the microbial load in the aquarium was not significantly affected by any individual hydrological parameters. However, the combined effect of the various parameters influenced the general health of the aquarium reared fish. Three important factors were derived based on the principal component analysis including water and atmospheric temperature, nitrite, dissolved oxygen and pH, which significantly altered the general water quality. The study indicated that sudden imbalances in nutrient parameters following resetting of aquaria form the major reasons for mass mortality, among all groups of fishes. The bacterial isolates from aquarium fish were identified as *Flavobacterium* (27%), *Vibrio* (18%), *Pseudomonas* (16%), *Alcaligenes* (16%), *Acinetobacter* (11%) and members of Enterobactericeae family (8%). *Serratia marcescens* was isolated for the first time, which was frequently involved in ulcerative lesions of fish. This species was found to be highly proteolytic in nature, and in view of the results of preliminary studies, it was selected for investigating its role in inducing pathogenicity in the experimental host, clown fish (*Amphiprion sebae* Bleeker). The lethal infective dose of the live cells was found to be equivalent to 1×10^5 cells per gram of fish. The extracellular products (ECPs) of *Serratia marcescens* were highly toxic for the experimental host. The infective dose of the ECPs was estimated as 2.67 µg per gram body weight of fish. The heat labile nature of the ECP proteins was established by the *in vitro* studies. The gelatinase and caseinase activities were reduced on heat treatment at 50^0C for 10 minutes. Based on SDS-PAGE studies, it was estimated that major proteins responsible for pathology had their molecular weights ranging from 11kDa to 100 kDa and that most of the proteins had molecular weights higher than 40 kDa. The antibiotic susceptibility pattern of the important bacterial isolates showed that gentamicin, streptomycin, tetracycline, nalidixic acid and ciprofloxacin were capable of inhibiting growth of more than 90% of the isolates tested. The effectiveness of marine natural compounds, antiseptics and autogenous vaccine in controlling infection due to *Serratia marcescens* was also investigated. The extracts of the sponges (*Sigmodocea carnosa*, *Callyspongia* and an unidentified species), as well as those of two seaweeds (*Gracilaria corticata* and *Valanopsis pachyderma*) had excellent inhibitory activity. Among antiseptics, formalin, acriflavin and malachite green were effective. The efficacy of immunisation using *Serratia marcescens* bacterin was proved by the 100% Percentage Relative Protection (PRP) obtained after 35^{th} and 50^{th} day of administration of bacterin.

CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF PLATES

GENERAL

INTRODUCTION

GENERAL INTRODUCTION

Aquarium fish keeping is the second largest hobby in the world next to photography (Kumar, 1998) and is a leading cash crop in many parts of the world, including the United States (Chapman *et al.*, 1997). The whole ornamental fish sector represents $250 million worldwide and 20 times this level, which is about $4 billion, if the related merchandise products such as aquariums, fish food, algae, gravel, etc. are taken into account (Ounaies, 1998). The world marine ornamental fish hobby and industry account for 70-100 tonnes per year against the marine food fish industry, comprising around 100 million tonnes per year (Dawes, 1998). Marine tropical ornamentals virtually represent an unlimited un-tapped field with a diminishing supply. Because of this reason, marine tropical fish are estimated at $400 to $500 per pound compared to the freshwater counterparts ranging from $35 to $60 per pound (Hoff, 1993).

Damselfishes (Pomacentridae) are among the most common ornamental reef fishes in tropical seas, comprising 320 species in all, with 268 species in the Indo-West Pacific regions alone. The ever increasing demand of these fishes led to overexploitation of some species necessitating conservation of the reefs (Sin *et al.*, 1994) and thus to the development and application of sustainable aquaculture techniques to ornamental marine species (Heslinga, 1996).

The demand for wild ornamental fish such as damsels remains strong since the success of captive fish breeding have been challenged by high mortality at early life stages (Nelson and Ghiorse, 1999). Similar is the case with many of the other genera belonging to Pomacentrids. The condition is strikingly in contrast to that of fresh water ornamental fish industry, about 90% of which are commercially reared for aquarium purposes and has been already established. The species of marine ornamental fish, which can be reared

reliably in large quantities, include a dozen anemone fishes, seven species of gobiids (Gobiidae), five species of cardinalfishes (Apogonidae) and eight species of pseudochromids (Pseudochromidae) (Arvedlund *et al.*, 2000). Whereas the aquaculture of fresh-water species is sufficient to respond to 95% of market needs, sea species are only now beginning to be farmed (Ounaies, 1998). Among these the best known are the various clownfish species, but even seahorses are now being bred commercially (Dawes, 1998)

As already stated, the disparity is also visible in the market price of marine and fresh water ornamentals. As reported by Gomes (1996), the price for marine fish can reach up to US $1000 per kg, while their counterparts earn about only one tenth of it. Thus, the marine ornamentals represent a low-volume, high-value industry, with the world's supply coming from around 150,000 sq. miles (Dawes, 1998).

It is observed that in spite of the huge demand for tropical marine ornamental fish throughout the world, the industry is dependent on the wild caught fish specimens captured from coral reefs, primarily in the developing Archipelagic nations of the Indo-Pacific region (Heslinga, 1996). Even for captive maintenance or rearing, there are inherent difficulties related to diet and diseases. With the exception of a few fishes, aquaculture and the hatchery production of these ornamental marine species remain at an experimental level. Thus, their rearing appears to be difficult and the experimental studies have not yet yielded proper rearing techniques (Ounaies, 1998). The development of commercial warm water marine fish farming is constrained by factors such as reproduction, nutrition, water quality, and economics (Tucker, 1993), all of which are valid in marine aquaria also.

Broodstock management and larval rearing are two critical stages as is the case with all fishes reared in captivity. Environmental conditions in the system during maturation have to be carefully monitored and tuned for successful spawning, which gives best results when good quality water is

provided. It is also noted that the fish, which are reared under captivity or those, which are acclimated to confinement, are likely to give good results (Gomes, 1996), which is not always practical with respect to the marine ornamentals whose rearing has been a recent activity. However, for varieties of high demand such as seahorses, various captive breeding ventures have been established to reduce the pressure on the natural populations (Lockyear *et al*., 1997; Kaiser *et al*., 1997). Broodstock nutrition is another decisive factor, which affects the spawning process, as well as the health of the critical initial larval stages (Gomes, 1996).

Larval rearing is time consuming and expensive with respect to the marine ornamentals (Arvedlund *et al*., 2000) and high mortality is rampant in their early life stages (Nelson and Ghiorse, 1999). Regarding larval nutrition, best food to be provided, both live feed and artificial feed are factors of importance that determine the success of the hatchery operations. Also, many of the causes of deformities including diseases and mortalities are related to nutrition.

Other than the above-mentioned factors, production failures very often result from diseases, which are due to infectious as well as non-infectious agents. The stress factors prevailing in the system induce physiological changes in the animals suppressing their immune system (Maule *et al*., 1989), making them increasingly susceptible to infectious agents (Pickering and Duston, 1983). With the introduction of new species for ornamental purposes, their capture and maintenance became intensified leading to competition to gain hold of the booming commercial market. The rapid commercialisation of the industry paved the way for entry of novel pathogens, which along with the improper management procedures became one of the major bottlenecks of production.

In marine aquaria, the important types of non infectious problems encountered include damages due to behavior activity, feed related disorders,

diseases associated with water chemistry, toxicities arising from use of treatment chemicals, damages due to handling and disorders related to weather conditions. The infectious diseases are due to biological agents including bacteria, parasites, fungi, viruses etc. and non-infectious agents.

Although the disease factor is found to play an important role in the success of aquariculture, the causes and solutions for the problem have not been studied in detail, except for information on non infectious diseases such as gas super saturation disorders, morphological and physiological changes resulting from imbalances in water chemistry and toxicities as well as infectious disorders including viral diseases such as lymphocystis (Nigrelli and Ruggieri, 1965; Bernoth and Mark St. J. Krane, 1995), studies on protozoan parasites (Lipton, 1993, Paperna,1980), reports of fungal diseases (Sindermann, 1990), acid fast bacteria (Giavenni *et al.*, 1980) and *Vibrio* and related bacterial infections (Love *et al.*, 1981; Nelson and Ghiorse, 1999; Sindermann, 1990).

Bacterial pathogens, because of high infectivity and short generation time produce severe effects on fish held in marine aquaria (Sindermann, 1990). The information on different aspects of diseases including host range, epizootiology, clinical signs, prophylaxis and therapy are ambiguous with respect to marine ornamentals. As stated earlier, the primary predisposing factors of diseases lead to build up of microbial load and very often provide favourable conditions for opportunistic pathogens to multiply. The normal bacterial flora, which are part of the environment, can become secondary invaders when the system is under stress and may lead to serious pathological conditions. Investigations on the common invaders gaining entry in this way in a special environment like the marine aquarium is of importance,considering its economic value, and the impact of diseases on the output.

The prevention of bacterial diseases in the aquarium is another area, which has not been given much attention. Most of the available treatment methods included applications with commonly available medications

irrespective of the exact role played by them. Successful control of fish diseases is a complex problem affected by water management, preventive treatments and appropriate application of chemotherapeutics (Plumb, 1992). Effective control measures have been developed for some of the significant pathogens, while some remain resistant to treatment (Sindermann, 1990). Many diseases of fish are secondary to environmental insult, and can often be prevented through maintenance of a high quality environment. Another approach to disease control is the use of prophylactic treatments when fish are handled. Prophylaxis in the from of quarantine, maintenance of good water quality and adequate nutrition are suggested as measures to prevent diseases (Sindermann, 1990). However, a combination of environmental manipulation and judicious use of chemotherapeutants forms the best approach. Vaccination of fish for disease prevention is becoming more popular around the world and with the use of improved administration methods, will eventually be a significant procedure in fish disease management (Newman, 1993)

Thus, the role of disease prevention and control in properly managing the marine aquarium can be appreciated; for which along with chemotherapy, recent developments including use of immunostimulants, probiotics, bioactive compounds, and immunoprophylaxis are recommended. Hence, the present study was carried out to understand the onset of bacterial diseases with regard to environmental influences, microbial load, and to identify the commonly occurring bacterial pathogens of marine aquarium, which are the causes for lesions as well as morphological, anatomical and physiological abnormalities. The work was also planned to study in detail the aspects of pathology of a selected pathogenic isolate and means of controlling the same by *in vitro* and *in vivo* studies.

In view of the limited earlier information and the prevailing lacuna particularly in the field of bacterial diseases of the marine ornamental fish, the thesis was initiated with the following objectives:

1. To record the physico-chemical as well as biological characteristics of the rearing environment and their possible influence on the health status of the common marine ornamental fish.
2. To record the mortality pattern together with external symptoms of the affected fish.
3. To isolate and characterise the important bacterial pathogens and to detect their susceptibility pattern towards the common antibiotic compounds.
4. To experimentally infect healthy groups of fishes and to detect the lethal influence of the important pathogenic isolates.
5. To determine the possible role of the exocellular products produced by the bacterial pathogenic isolate.
6. To determine the role of autogenous vaccines in protecting or providing resistance to fish when challenged with the potential pathogenic isolate.

CHAPTER 1

STUDIES ON THE INFLUENCE OF REARING CONDITIONS ON THE ONSET OF DISEASES AMONG AQUARIUM HELD MARINE ORNAMENTAL FISHES

Diseases in aquaculture are production bottleneck factors of crucial importance, which decide the fate of commercial fish rearing industries including marine aquaria. With the introduction of new species for ornamental purposes and due to the ever increasing demand, their capture and maintenance became commercialised, which led to the introduction of novel pathological conditions. The disease conditions often result due to the stress factors associated with intensification of rearing and improper management prevailing in the confined systems.

The various hydrological parameters as well as microbial load in the system have a bearing on the status of health of the fish. Significance of congenial environmental conditions in the well being of the captive fishes is well documented in aquaculture. Researchers recently emphasised the importance of physicochemical parameters in causing infections by microbes and outbreak of diseases. It was recorded by Pickering and Duston (1983) that commonly encountered stressors suppress the immune system of teleosts and make them less resistant to infection. Water instability (fluctuation of water quality parameters, elementary chemistry changes, variation in microbial and plankton community etc.) is considered by experts to be the major factor promoting diseases and mortalities (Gomes, 1996). Speare (1998) stated that water chemistry and quality problems in aquaculture are more significant to morbidity and mortality than all infectious disorders combined. According to Plumb (1992), environmental manipulation of fish rearing water will help to control many of the stress-mediated diseases.

Considering these aspects, the present study was conducted to evaluate the role of environmental factors in the health of aquarium held tropical

marine ornamental fish. The system under observation was the marine aquarium of Vizhinjam research centre of C. M. F. R. I., which was started with the objective to maintain and to study the biology and breeding of commercially important marine ornamental fishes available in the coast. Besides, collection of ornamental fishes was periodically conducted for maintaining in the aquarium. The fishes were maintained in FRP tanks of 1 tonne to 5 tonne capacity, 1 tonne cement tanks, as well in glass aquaria of 8×8×2 feet, all of which were provided with facilities for biological filtration. Consequently, the present study may be considered as the monitoring of the general health aspects of the marine ornamental fishes under captivity. The observations on the water quality conditions prevailing in the system, bacterial load as well as trend of deaths of important groups were taken.

Role of Hydrological Parameters in Disease Occurrence

Water quality is the key factor in confined systems such as aquaria, which is mostly controlled by culturist himself and is often the reason for disorders in the system. The important factors, which affect the health of the marine ornamental fish or its performance, include water quality, temperature, diseases and season (Gordin, *et al.*, 1990). In their natural habitat such as specialised environments like coral reef ecosystems, with good water quality, the marine ornamentals are known to live in close association with invertebrates (Pichai Sonchaeng, 1988). A high quality of water in the system is always advised while maintaining them under captive conditions. However, deterioration in water quality and resulting difficulties are widespread in marine aquaria. With fluctuations in the water quality criteria, the fish tries to adapt itself, by physiological responses, which finally leads to the condition called "stress" to the animal.

Fish are easily stressed and this state can be brought about in many ways, such as by deterioration in water quality, disease, social interaction amongst the fish, crowding etc. Bry (1988) has described deterioration of water

quality, as one of the potential factors of stress. Stress affects fish in many ways, reducing their overall performance (Sumpter, 1993).

In marine aquaria, it is not uncommon to have declining water quality, which cause outbreak of diseases. In spite of the precautions taken, water systems commonly in use for aquarium are subject to contamination by many inorganic and organic compounds, as well as nitrogen waste components, in particular ammonium, nitrites, and nitrates, which at certain concentrations become highly toxic (Hiatt, 1998). Combinations of environmental and host factors are cited as causing similar range of disease outbreaks in intensively reared species (Mellergaard and Dalsgaard, 1987). How the different factors affect the host pathogen environment interaction decides the occurrence of diseases.

Oestmann (1987) suggested disease in the home marine aquarium might occur spontaneously, by accidental introduction, or as the result of faulty management. These management failures are considered as the biggest source of disease in the home aquarium. Plumb (1992) reported that many diseases of fish are secondary to environmental insult, and can often be prevented through proper management or environmental manipulation.

One of the fundamentals in preventing fish diseases is monitoring of water quality parameters, such as dissolved oxygen, ammonia, and nitrite (Avault, Jr., 1997). According to Plumb (1992), maintenance of a high quality of water, which is free of pesticides and other pollutants, along with proper management of water quality is essential for disease control. Handy and Poxton (1993) described that success in intensive aquaculture requires the maintenance of good water quality. They observed that dissolved oxygen (DO) levels greater than 90% saturation, water pH values between 6.0 and 9.0, depending on the cultured species, and concentrations of suspended solids below 15 mg/l were preferable in culture systems. In addition, sufficient water flow to minimise the deleterious effects of oxygen consumption, carbon dioxide

and ammonia excretion by the fish on water quality were recommended by the same authors.

Bacterial infections in fish are secondary problems relating to the stresses of intensive culture such as temperature change, handling, breeding, poor water quality, parasite load, shipping, and even chemotherapeutic treatment. These factors are known to interfere with the immune response, resulting in decreased efficacy in both the humoral and cellular responses and phagocytosis (Dixon, 1991).

Hargis, Jr. (1991) reported that most of the eye diseases in finfish including exophthalmia, cataract, neoplastic changes, corneal and retinal lesions were exacerbated by stresses associated with culture, including poor water quality viz. gas imbalances, overcrowding and aggressive behavior.

Reproductive performance of the fish is also found to be influenced by the environmental stressors. Species may differ in the nature of their physiological response and reproductive cycles to the particular stressors applied to it. It is seen that timing of reproductive events including maturation and ovulation are influenced by other physiological variables responsive to stressors (Schreck *et al.*, 2001). Deterioration in water quality adversely affects even hardy fish like tilapias. A number of diseases like gill hyperplasia, presumably and principally due to chronic ammonia and nitrite toxicity, was common in recirculated fresh and brackish water culture systems (Lightner *et al.*, 1988).

Recirculating systems are considered as important habitats for multiplication of pathogenic microflora where stressful conditions such as poor water quality or high stocking densities, may contribute to disease outbreaks. In such systems, opportunistic microorganisms may cause diseases due to favorable conditions and the biofilters used may become reservoirs of pathogens (Noble, 1996). Non infectious problems, including high levels of

ammonia, nitrite, carbon dioxide, suspended solids, or ozone residual levels which predispose the fish to diseases such as those caused by bacteria (bacterial gill disease, furunculosis, bacterial kidney disease, fin rot), parasites, fungi (*Saprolegnia*), and viruses are also reported to cause mortalities in recirculating systems (Noble and Summerfelt, 1996).

As reported by Crosby (1996), between 1993 and 1995, out of the 27 fish health cases processed from recirculating systems, in Virginia, where ornamental fish was a major group, water quality was found to be a prevalent problem constituting about 29% out of a total of 151 cases submitted to the Virginia State University Aquaculture Diagnostic Laboratory.

As already stated, the extensive fluctuations in water quality parameters bring about conditions of stress to the animal, which result in physiological disorders and other pathological changes. The overall performance of the fish is affected due to the suppression of immune system.

Temperature

Increases in temperature adversely affect the health of fish and other aquatic life by lowering the dissolved oxygen content of the water, increasing the oxygen demand by increasing the metabolic respiratory rate, increasing the toxicity of harmful substances dissolved in water, favouring growth and invasiveness of bacterial pathogens and causing a variety of physiological responses in fish, some of which apparently lower disease resistance (Wedemeyer *et al.*, 1999).

Temperature was reported to affect the immune response of sunshine bass (Hrubec, *et al.*, 1996). Temperatures of 10 ^{0}C and 18 ^{0}C decreased the magnitude and delayed the time of the antibody response to *A. salmonicida* in sunshine bass.

Hyperplastic epidermal conditions were found to be associated with low temperatures in salmonids in Scotland, which was suspected to be due to extreme sensitivity to ammonia at low temperatures (Roberts, 1989).

Dissolved oxygen

In intensive culture systems, along with changes of ammonia content, dissolved oxygen has vital roles in the well being of the animals. In their review Braaten and Hektoen (1991) stressed the important role of environment in fish health management in the Asia Pacific region. They indicated that dissolved oxygen and ammonia are the two most important environmental factors to monitor. According to Supamataya (1988), dissolved oxygen and water temperature were main factors other than pesticide residues in water.

In confined aquarium fish, the gas bubble disease is associated with super saturation of water with nitrogen or oxygen (Roberts, 1989). Small marine fish larvae are significantly more sensitive to gas super saturation (Colt *et al.*, 1987). Exposure to gas super saturated water can cause epidermal erosions overlying dermal gas bubbles (Speare, 1991).

Occurrence of bacterial infections could be related to water chemistry parameters. The streptococcosis disease was produced experimentally in tilapia hybrids and the susceptibility was correlated to low dissolved oxygen levels and high nitrite concentrations (Bunch and Bejerano, 1997). Both the stress factors proved to increase the mortality. No additive effect was seen when these factors were applied simultaneously. It was found that *Streptococcus* spp. are opportunistic pathogens and they depend on stress to assert pathogenicity.

No probable upper limit for dissolved oxygen is recommended, but generally the lower limit is set at 4 ppm for warm water species and 5 ppm for salmonids (Wedemeyer *et al.*, 1999).

pH

It was indicated by Roberts (1989) that factors, which cause a rapid change of pH, are deleterious to the gills. A pH range between 5 and 9 is normally not toxic to most fish species, except in presence of aquatic contaminants like metals (Witters, 1998). Most fish species are susceptible to mortality, impaired growth and reproduction below pH values of 5.0 (Freda and McDonald, 1988). Morphological changes especially that of the gills and physiological changes associated with ammonia and urea metabolism are found to be affected by alkaline pH (Wood, 1993; Laurent *et al.*, 1995). Lowered pH is believed to increase the susceptibility to diseases, but this condition is absent in the strongly buffered sea water (Wedemeyer *et al.*, 1999).

The content change of dissolved oxygen, pH and ammonia in seawater were found to have much effect on normal growing of the economic fishes. When the content of dissolved oxygen was kept above 4ml/l , pH at about 8 and NH_4^+ N at 0.05,the fish were found to grow normally (Wang and Zhao, 1995).

Ammonia, nitrate and nitrite

Ammonia, nitrate and nitrite are significant factors determining the health of confined fishes. The physiological effects of ammonia and nitrite toxicity in teleosts are yet uncertain and controversial (Speare, 1998). The NH^{4+} ions are relatively harmless to fish, but ammonia is quite toxic, especially to salmonids and the toxicity is determined by the pH of water containing ammonia or ammonium salts. Fish can tolerate a high level of NH^{4+} at low pH, but become more sensitive as pH increases and toxic NH_3 is formed. Other factors, which influence the ammonia toxicity, include dissolved carbon dioxide and oxygen concentration. Presence of carbon dioxide brings down pH and decreases the susceptibility to NH_3 toxicity. But if the dissolved oxygen concentration is low, the amount of carbon dioxide given off is decreased, resulting in high pH of water, which increases the ammonia toxicity. Ammonia

toxicity results in the inability of haemoglobin to bind oxygen (Wedemeyer *et al.*, 1999).

Branchial lesions due to ammonia exposure were reported by Smith and Piper (1975), Smart (1976) and Thurston *et al.* (1984) though other workers (Daoust and Ferguson, 1984, Lang *et al.*, 1987) could not repeat the effects.

The elevated levels of nitrate decreased magnitude and delayed the time of the antibody response to *A. salmonicida* in sunshine bass (Hrubec, *et al.*, 1996).

In addition to the chemical parameters, the toxicity due to ammonia, nitrite and nitrate content are often related to the presence of colonising nitrifying bacteria. One of the reasons for petechial hemorrhages in zebra fish after four weeks of stocking was detected to be due to poor water quality in tanks with toxic concentrations of nitrite levels of 1 to 5 ppm. Because the housing system had not been seeded with *Nitrobacter* spp. and *Nitrosomonas* spp. sufficiently prior to the arrival of the fish, a lack of colonising nitrifying bacteria was deemed to be the cause of the high nitrite level, which, along with over crowding, stressed the fish and increased their susceptibility to motile aeromonad septicaemia. Reduction of nitrite level and stocking density avoided such septicaemic conditions (Pullium *et al.*, 1999).

Nitrite toxicity, can affect salmonids (Lewis and Morris, 1986). Nitrite induced death may stem from a number of factors like methemoglobin production by chemical oxidation of haem which results in a hemoglobin incapable of combining with oxygen, leading to hypoxia.

Pollution and diseases

Although the link between adverse water quality and fish diseases is not proven, the end results, of presence of pollutants in the environment,

include immunosuppression, reduced metabolism, epidermal papilloma, fin/tail rot, gill disease, hyperplasia, liver damage, neoplasia and ulceration (Austin, *et al.*, 1999).

The effects of pollution in fish also include lesions attributed to *Serratia plymuthica*, fin and tail rot caused by *Aeromonas hydrophiia* and *Pseudomonas fluorescens*, gill disease resulting from the activity of *Flavobacterium* spp., vibriosis as caused by *Vibrio anguillarum*, and enteric redmouth (caused by Yersinia *ruckeri*) etc. (Austin *et al.*, 1999). Studies by the same authors indicated higher than usual quantities of organic material, oxygen depletion, changes in pH values and enhanced microbial populations resulted in diseases caused by *Aeromonas*, *Flavobacterium* and *Pseudomonas*.

MATERIALS AND METHODS

Sample Collection

Collection of samples at predetermined intervals from the marine aquarium, Vizhinjam research centre of C. M. F. R. I. was carried out to monitor water quality and to evaluate the bacteriological load.

General features of the marine aquarium: The aquarium, which was monitored for estimating hydrological parameters and microbial load, was an all glass tank fabricated in the aquarium with a dimension of 8×2×2 feet. The aquarium was provided with filter bed and airlift facility for water recirculation rate of 24 litres per minute (1440l/h). In addition,water exchange at the rate of $1/5^{th}$ the volume was done once in two days after removing the waste accumulated over the filter bed. The filter bed was of 3 inches thickness comprising coral sand and coral stone laid on perforated perspex bottom. The filtered water was recirculated via PVC tubes by providing aeration by stones.

The stocking density in the aquarium tanks depended on the size and the behavioral aspects of the fish. The common ornamental groups maintained in the tank included Pomacentrids, Chaetodontids, Serranids and miscellaneous groups of fishes.

General health conditions of fish in the aquarium: The confined fish in the aquarium showed normal swimming and feeding activity. Since the fishes were introduced to the aquarium following a period of quarantine for acclimatisation, the fishes usually showed normal behaviour under hygienic conditions.

Sampling for water analysis: Temperature of the aquarium water and atmosphere were recorded with thermometer (sensitivity of ±0.1). Samples for

other determinations such as salinity, ammonia, and nitrate estimation were collected in plastic bottles. For estimating dissolved oxygen, samples were collected in 125 ml glass stoppered reagent bottles without entangling air bubbles. The sample was fixed immediately by adding Winkler A (composition: 20g manganese chloride in 100 ml distilled water) followed by Winkler solution B (composition: 41g of sodium hydroxide, 25g of potassium iodide in 100 ml distilled water). Sampling for water quality was carried out every fortnight.

Sampling for total microbial load: In order to estimate the total bacterial load by the Total Plate Count (TPC) method, samples were collected in sterile bottles at fort-nightly intervals. The average values for three months corresponding to premonsoon, monsoon, post monsoon and transition were computed for all the water quality parameters and microbial load.

Estimation of Hydrological Parameters

Salinity: Estimation of salinity was done following the Mohr's titration method described by Strickland and Parsons (1968).

Dissolved Oxygen: Dissolved oxygen estimation was done by the Winkler's method (Strickland and Parsons, 1968).

pH: pH of the sample was measured by means of a digital pH meter (make: Elico, India), having a glass electrode and a reference electrode. Before taking pH, the instrument was calibrated with pH buffer solutions of pH, 4.0, 7.0 and 9.0.

Ammonia: Total ammonia was estimated by the phenol hypochlorite method as described by Strickland and Parsons (1968). The intensity of blue colour developed as a result of the reaction was measured by the spectrophotometer at a wave length of 643 nm. Freshly prepared working standards were prepared

from the standard stock solution of ammonia and a standard graph was drawn, from which the ammonia content was computed.

Nitrate and nitrite: Nitrate and nitrite content was estimated by the Morris and Relay method as described by Strickland and Parsons (1968). For estimation, with working standards of the respective standard stock solutions, standard graphs were prepared and the concentrations of sample were estimated from the graph.

Microbial Load of Aquarium Water

Total Plate Count: For estimating the bacterial load by total plate count method, serial dilution technique was followed. Aged seawater was used as the dilution medium. All glassware and dilution medium were sterilized by autoclaving at 121^0 C for 20 minutes. Dilutions up to 10^7 were prepared and inoculated in sterile Zobell marine agar medium (HiMedia) by the pour plate method. Triplicates were maintained.

Mortality Pattern in the Aquarium

The occurrence of mortality in the marine aquarium was monitored for a period of seven months from March 1999 to September 1999 to know the general pattern of mortality with respect to the fluctuating hydrological parameters as well as total microbial load in the system. The study also had the objective of finding out the common groups which were susceptible.

In addition, general observations on mass mortality and occurrence of sudden deaths in the marine aquarium were taken.

Determination of Correlation between water quality and incidence of disease by Statistical analysis

In order to correlate the occurrence of diseases and mortality in the aquarium with the prevailing hydrological conditions, an index of water

chemistry incorporating the different water quality parameters was constructed by the principal component analysis. Three important factors were extracted by factor analysis using the SYSTAT-7 package. The index was tested against microbial load as well as mortality pattern of important groups of fish and the general mortality in the aquarium.

RESULTS

Water Quality Conditions in Marine Aquarium

The results of analysis of water samples collected for estimation of water quality parameters as well as microbial load indicated a positive influence of these factors in the disease/mortality pattern of fishes in the aquarium as well in the microbial load of the system. The average values for three months corresponding to premonsoon, monsoon, post-monsoon and transition during March 1999 to August 2000 are given in Table 1.

Trend in the atmospheric and water temperature for the period of study is given in Fig.1. The pH values recorded in the aquarium during the period of study are given in Fig. 2.

From these figures,it can be seen that there were less fluctuations in temperature in the aquarium, both atmospheric temperature (standard deviation-1.44) and water temperature (S.D.-1.18) as well as in pH (S.D.-0.09), which fluctuated between 7. 30 and 7.70.

Salinity in the marine aquarium fluctuated over the period of observation with a standard deviation of 1.38 whose values are shown in Fig. 3.

In Fig. 4, the fluctuations in the dissolved oxygen content of the aquarium are given. The variations in values of dissolved oxygen in water showed a standard deviation of 1.23.

Table 1. Average values (for three months) of water quality parameters and microbial load in marine aquarium during March 1999 to august 2000

Period	Atm. Temp. (°C)	Water Temp. (°C)	DO (mg/L)	Salinity (ppt)	pH	Nitrate (µgat/L)	Nitrite (µgat/L)	Ammonia (µgat/L)	Microbial load (CFU)
March-May	29.5	29.3	5.10	34.21	7.46	7.34	0.14	0.67	34.66×10^{2}
June-August	28.0	27.8	5.64	33.96	7.49	13.47	0.24	0.49	39.35×10^{2}
September -November	28.8	27.9	5.63	33.19	7.47	14.14	0.17	0.55	73.46×10^{2}
December-February	28.7	28.3	7.37	33.28	7.60	12.82	0.05	0.04	9.86×10^{2}
March-May II	31.1	30.2	6.34	32.24	7.59	9.37	0.006	0.04	19.9×10^{2}
June-August II	29.7	28.1	7.06	34.59	7.60	19.14	0.26	0.43	148.33×10^{2}

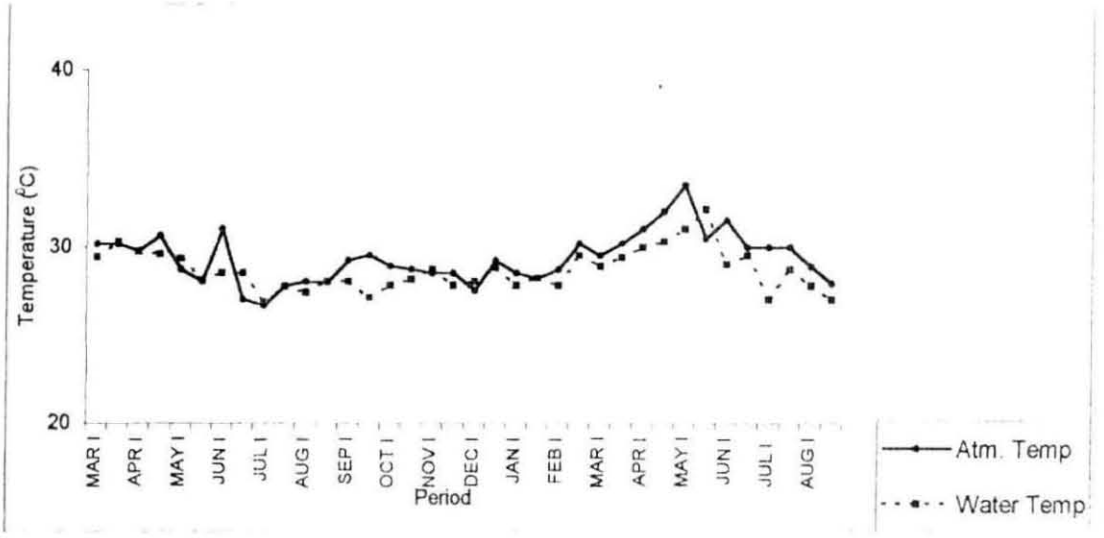


Fig. 1. Temperature changes in the marine aquarium

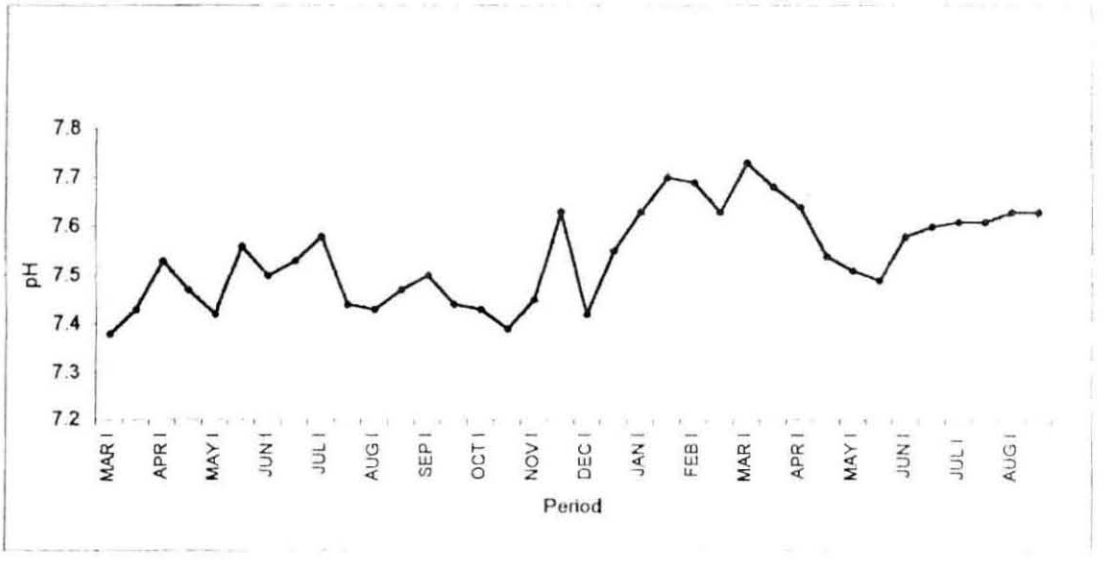


Fig. 2. pH changes in the marine aquarium

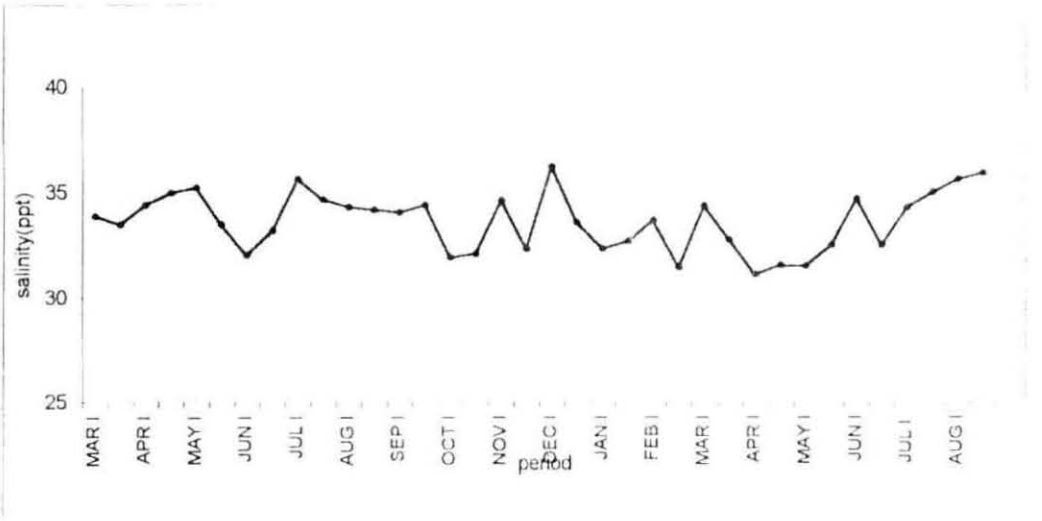


Fig. 3. Salinity changes in the marine aquarium

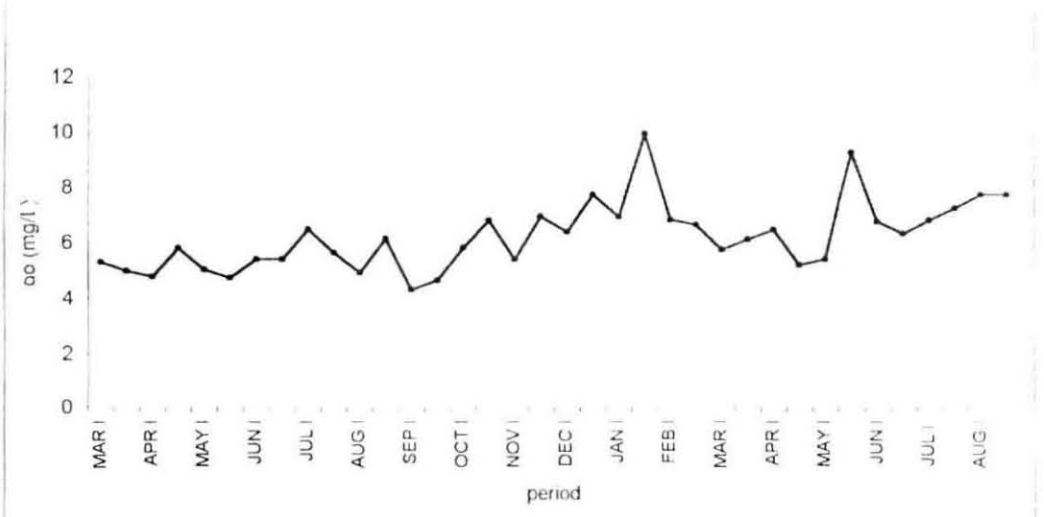


Fig. 4. Trend of dissolved oxygen content in marine aquarium

Ammonia, nitrate and nitrite

These three factors significantly influenced the mortality pattern of the marine ornamental fishes. Though the values were maintained low because of biological filtration, any slight increases resulted in the mortality of the fishes. The total number of fishes died during the observation period followed a pattern similar to that of ammonia content of the aquarium water over the same period (Fig. 5).

Fluctuations in ammonia (S.D.-0.12) and nitrite (0.39) content in water were similar as depicted in Fig. 6. The ammonia content was always higher than nitrite levels.

Nitrate content in the aquarium water was one factor, which showed wide fluctuations during the period of study with a standard deviation of 6.34. The variations in nitrate levels are shown in Fig.7.

Total Microbial Load

The bacterial load of water as depicted in Fig. 8 indicated wide fluctuations. It had no significant correlation with the hydrological conditions, as evident from the correlation chart (Table 2) between the water chemistry index and microbial load.

Correlation of Water Chemistry Index (WCI)- Extraction of Principal Components by Factor Analysis

From statistical factor analysis conducted with hydrological parameters, three principal factors affecting the water chemistry were estimated. The results (factor loadings) are given in Table 3.

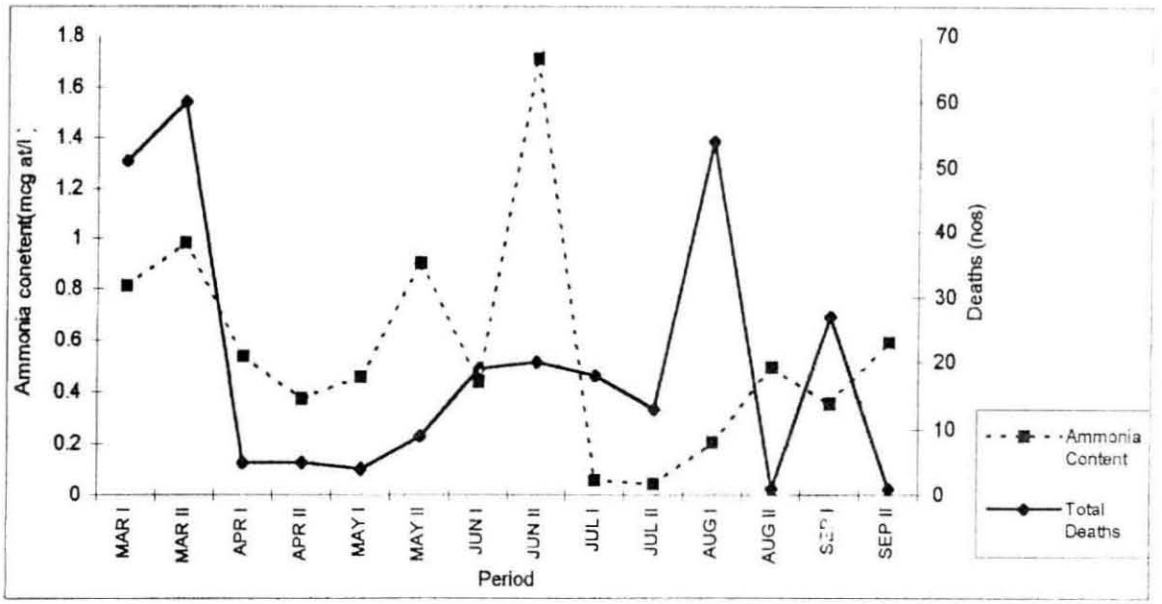


Fig. 5. Fluctuations in ammonia content and total number of deaths in the aquarium

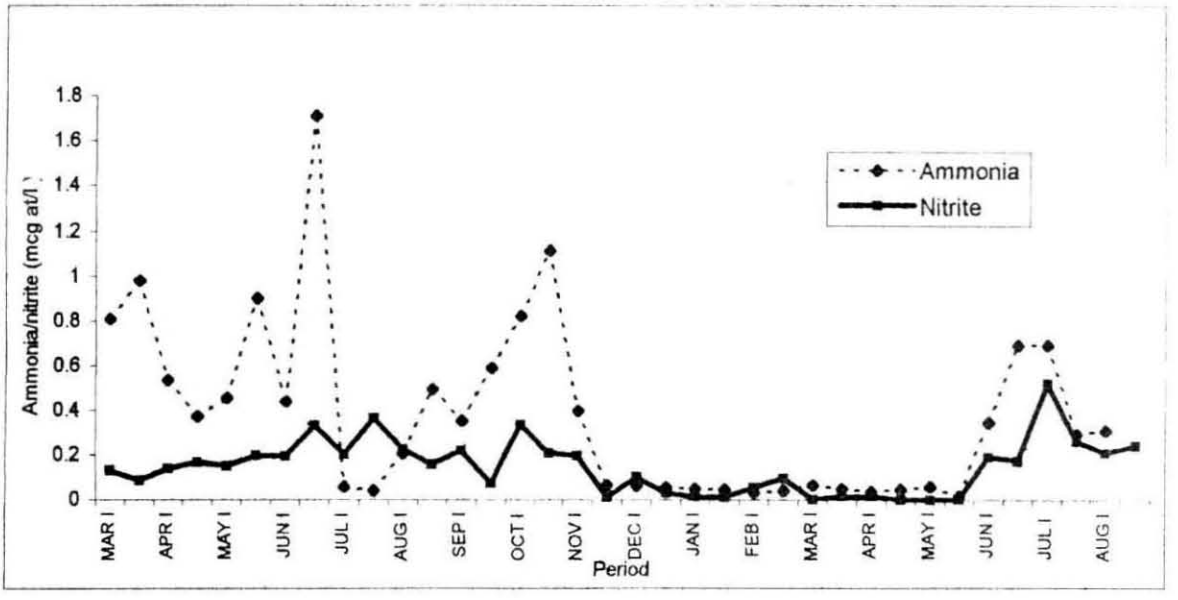


Fig. 6. Ammonia and nitrite content in aquarium water

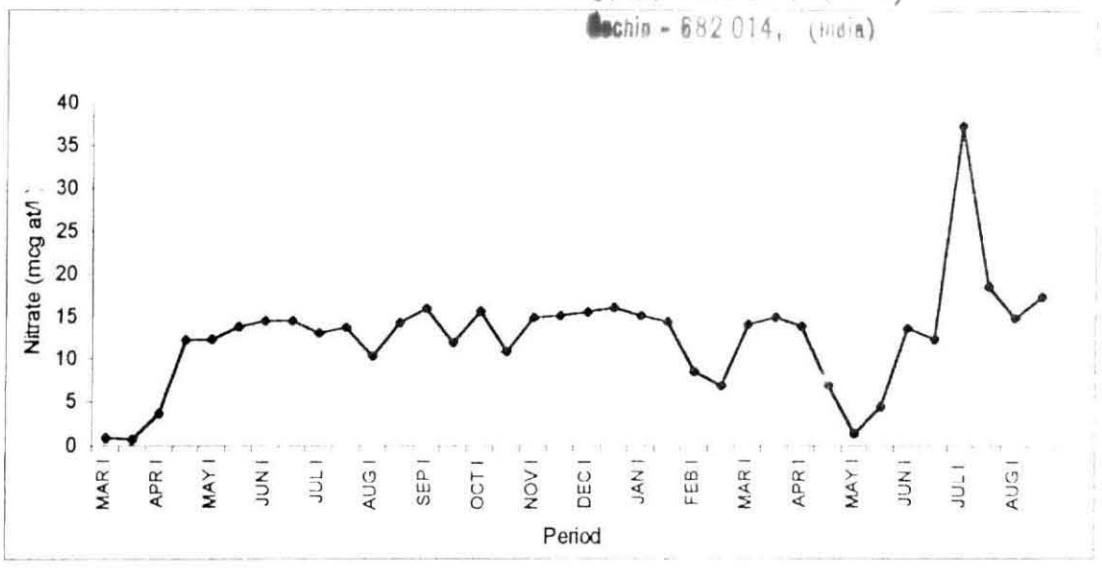


Fig. 7. Trend of nitrate content in aquarium water

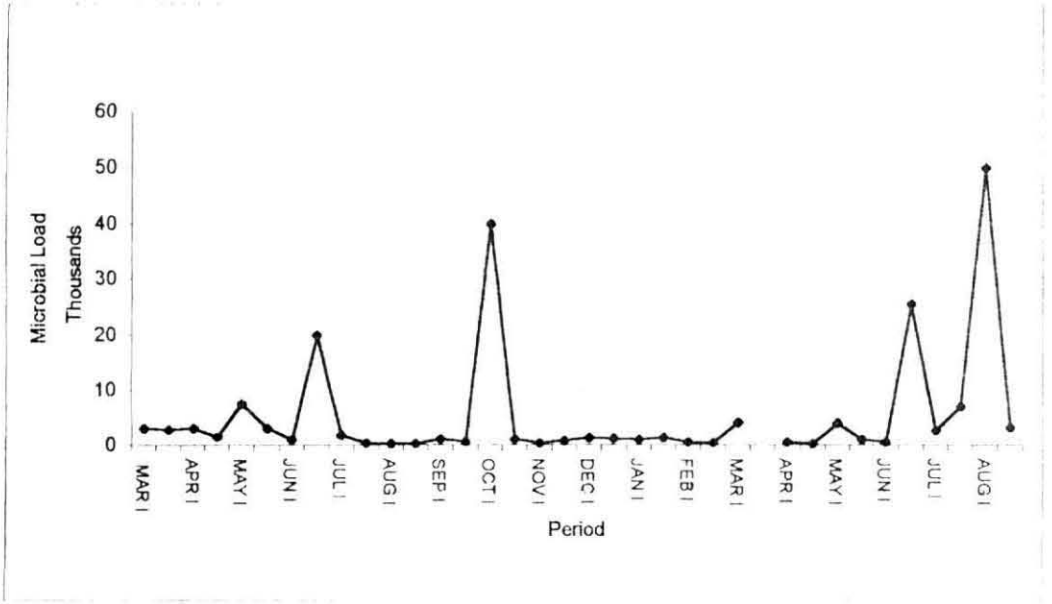


Fig. 8. Total microbial load of water in the aquarium

Table 2. Correlations of water quality parameters, microbial load and WCI factors

(Marked values indicate significant correlation at $p < 0.05$)

	Atmospheric Temperature	Water Temperature	Dissolved Oxygen	Salinity	pH	Nitrate	Nitrite	Ammonia	Microbial Load	Factor 1	Factor 2	Factor 3
Atmospheric Temperature	1.0	0.70	-0.095	-0.473**	0.051	-0.306	-0.326	-0.182	-0.098	-0.74**	-0.147	-0.37*
Water Temperature	0.70*	1.0	0.017	-0.441**	-0.081	-0.61**	-0.504**	-0.095	-0.096	-0.849**	-0.245	-0.074
Dissolved Oxygen	-0.095	0.017	1.0	-0.062	0.474*	0.212	-0.185	-0.368*	0.079	-0.099	0.757**	-0.064
Salinity	-0.473**	-0.441**	-0.062	1.0	-0.146	0.26	0.372*	-0.008	0.029	0.633**	0.037	0.563**
pH	0.051	-0.081	0.474**	-0.146	1.0	0.306	-0.262	-0.417*	0.056	-0.138	0.819**	-0.248
Nitrate	-0.306	-0.61**	0.212	0.26	0.306	1.0	0.564**	0.023	0.111	0.676**	0.443**	-0.437**
Nitrite	-0.326	-0.504**	-0.185	0.372*	-0.262	0.564**	1.0	0.51**	0.31	0.795**	-0.252	-0.367*
Ammonia	-0.182	-0.095	-0.368	-0.008	-0.417*	0.023	0.51**	1.0	0.324	0.376*	-0.668**	-0.389*
Microbial Load	-0.098	-0.096	0.079	0.029	0.056	0.111	0.31	0.324	1.0	0.205	-0.049	-0.244
Factor1	-0.74**	-0.849**	-0.099	0.633**	-0.138	0.676**	0.795**	0.376*	0.205	1.0	0	0
Factor2	-0.147	-0.245	0.757**	0.037	0.819**	0.443**	-0.252	-0.668**	-0.049	0	1.0	0
Factor3	-0.37*	-0.074	-0.064	0.563**	-0.248	-0.437**	-0.367*	-0.389*	-0.244	0	0	1.0

Table 3. Principal factors of water chemistry

Statistical factor analysis	Factor loadings (unrotated) (Marked loadings are > 0.70)		
Variable	Factor 1	Factor 2	Factor 3
At. Temperature	-0.7395 *	-0.1467	-0.3700
Water temperature	-0.8488 *	-0.2449	-0.0742
DO	-0.0989	0.7573 *	-0.0642
Salinity	0.6325	0.0368	0.5627
pH	-0.1381	0.8191 *	-0.2475
Nitrate	0.6757	0.4432	-0.4367
Nitrite	0.7948 *	-0.2516	-0.3672
Ammonia	0.3761	-0.6675	-0.3885

An index of water chemistry incorporating all the factors studied was obtained by factor analysis, viz. factor 1, factor 2 and factor 3 which were found to be the principal factors influencing the water chemistry among all the parameters studied. In Factor 1, the important variables determining the water chemistry were found to be declined values of atmospheric and water temperature and increased values of nitrite. In addition, salinity and nitrate was found to influence the first factor. For Factor 2, increased values of dissolved oxygen and pH were the main contributing parameters along with ammonia values. In Factor 3, the major effect was due to variations in salinity. It can be concluded that the above mentioned factors decided the general water quality of the marine aquarium (Table 3).

Correlations of Environmental Parameters and Microbial Load

Microbial load

The main factors showing positive correlation with bacterial load in the system were ammonia, nitrite and nitrate, along with factor 1 of the water chemistry index (WCI). However, the total microbial load was not affected significantly by any of the water quality parameters. Factor 3 of the index showed a slight negative correlation.

Ammonia, nitrite and nitrate

The fluctuation of these three parameters in the system was found to affect the overall health status of the aquarium-reared fish. These factors were in turn influenced by other physicochemical characteristics as well as microbial load.

The ammonia values exhibited a significant positive correlation with that of the nitrite content as well as factor 1 (constituted by lower values of temperature and nitrite content). In addition, a significant negative correlation

was obtained with that of dissolved oxygen content, pH, factor 2 and factor 3 in the system.

Nitrite content was positively correlated with salinity, nitrate, ammonia, and factor 1 whereas significant negative correlation was exhibited with water temperature and factor 3.

Nitrate concentration of the aquarium water showed significant positive correlation with factor 1 and factor 2. However, a significant negative correlation was observed with water temperature and factor 3 of the water chemistry index (WCI).

The correlation coefficients (r) of different water quality parameters, microbial load and the water chemistry index factors 1, 2 and 3 are given in Table 2.

Pattern of Disease Occurrence (Mortality Pattern) in Marine Aquarium

The mortality pattern in the aquarium as observed for a period of seven months also indicated that the microbial load as well as the water chemistry index influenced the health of the aquarium held ornamentals. The general mortality pattern in the aquarium for important groups of ornamentals like pomacentrids, acanthurids and chaetodontids along with trend of total deaths is given in Fig. 9.

From the correlation chart given in Appendix 1, it can be inferred that the total number of deaths in the aquarium had significant negative correlation with the nitrate content in the system. Deaths of pomacentrids also showed a negative correlation with the same factor as well as with Factor 2 of the water chemistry index. Another important finding was that during the same period, the microbial load in the system showed a highly significant positive correlation (at 0.01 level) with ammonia level of the water.

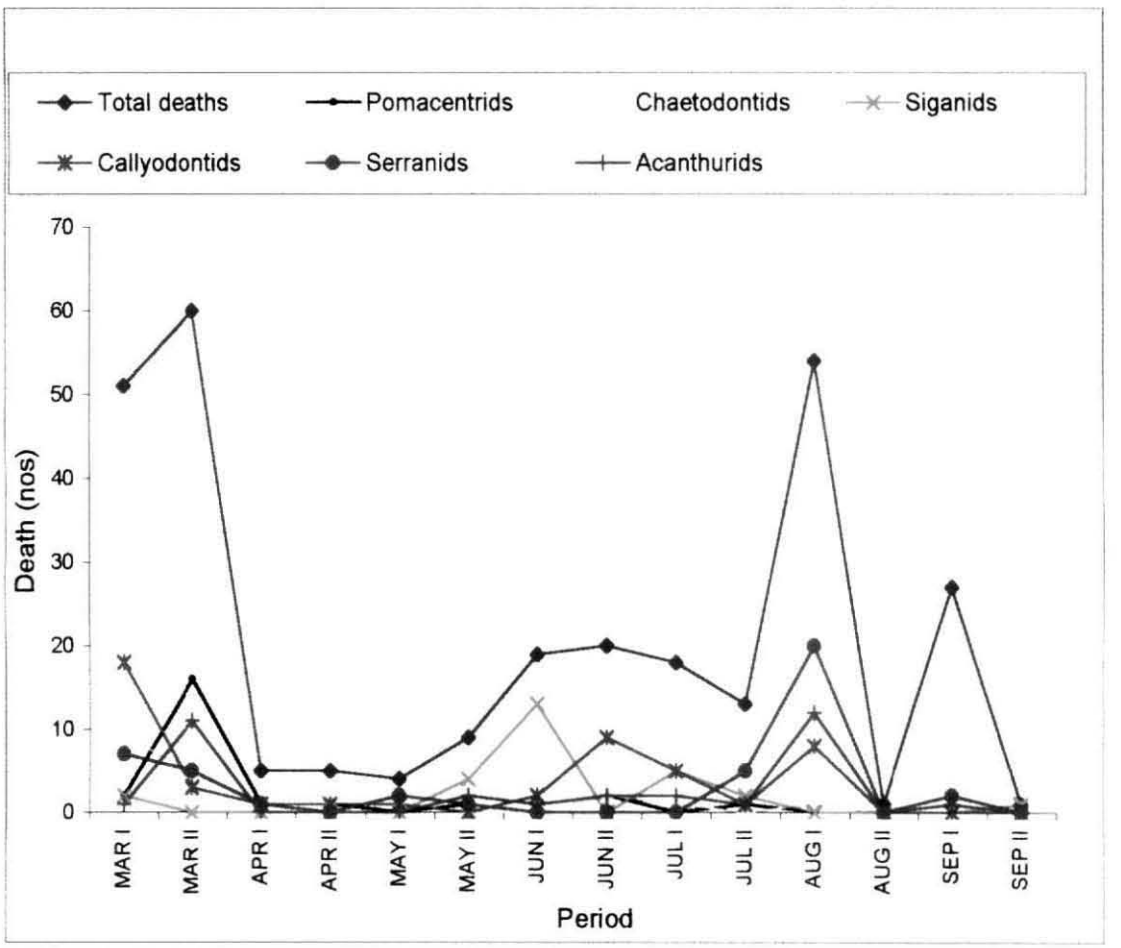


Fig. 9.Trend of fish deaths in the marine aquarium

The influence of the water chemistry index with the general death pattern in the aquarium is depicted in the Fig. 10, 11 and 12. Fig. 10 shows the trend of deaths in the aquarium with respect to the factor 1 of water chemistry index.

It can be seen from the figures that deaths of aquarium fish occurred with slight changes in the factor1 of water chemistry index. The lower values of factor 1, which was influenced by temperature and nitrite content of water was found to have a minor role in deciding the death pattern, which is also evident from the correlation chart (Fig 10).

The factors 2 and 3 had association with the death pattern in the aquarium as seen in Fig. 11 and Fig. 12.

From Fig 11 it is clear that more number of deaths of the aquarium fish occurred at lower values of Factor 2. Higher values of Factor 3 (especially salinity, nitrate and ammonia) corresponded with more number of deaths of fish to some extent (Fig. 12).

The external symptoms of samples representing two important species of ornamental fishes, which exhibited mortality, are shown in plates 1, 2, 3 and 4.

Occurrence of Mass Mortality in the aquarium

In the first week of November, 1998 high mortality of ornamental fish was noticed. Groupers started dying first followed by siganids, chaetodontids, mainly banner fish and eels, along with clown fish. The hydrological parameters at this period showed that water temperature was about 24.8 ^{0}C, dissolved oxygen 5.87 mg/l salinity 36.06‰ and pH within the normal range of 7.40 to 7.60. The mortality of clown fish persisted till the end of November, during which time dissolved oxygen levels were comparatively less (average values above 4.6 mg/l) and salinity ranged from 35 to 36.25‰.

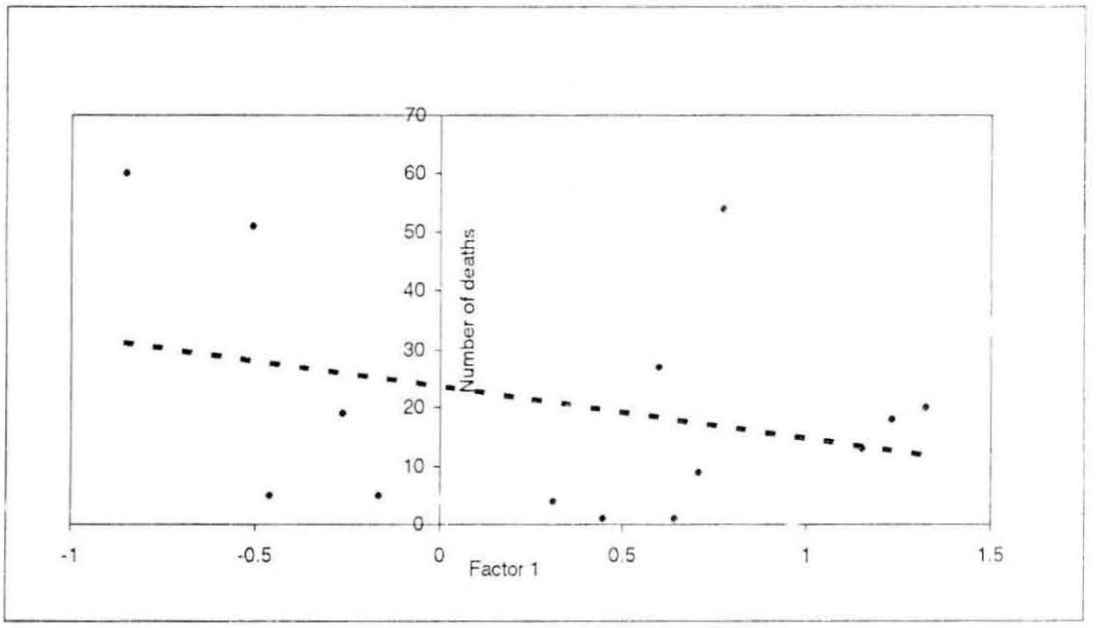


Fig. 10. Correlation of death trend with factor 1 of water chemistry index

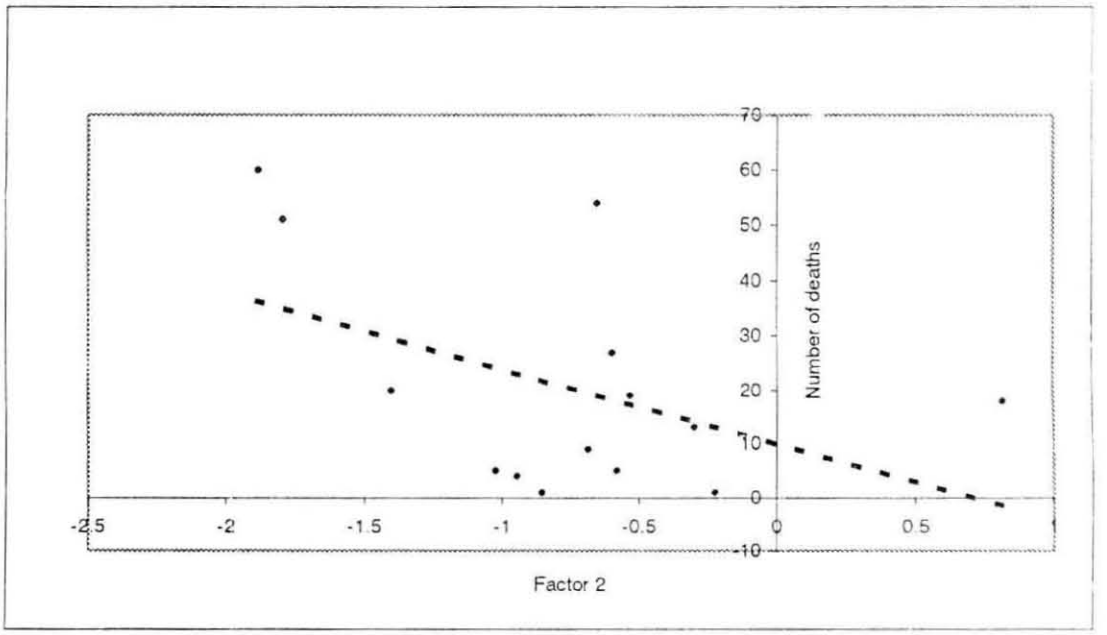


Fig. 11. Correlation of death trend with factor 2 of water chemistry index

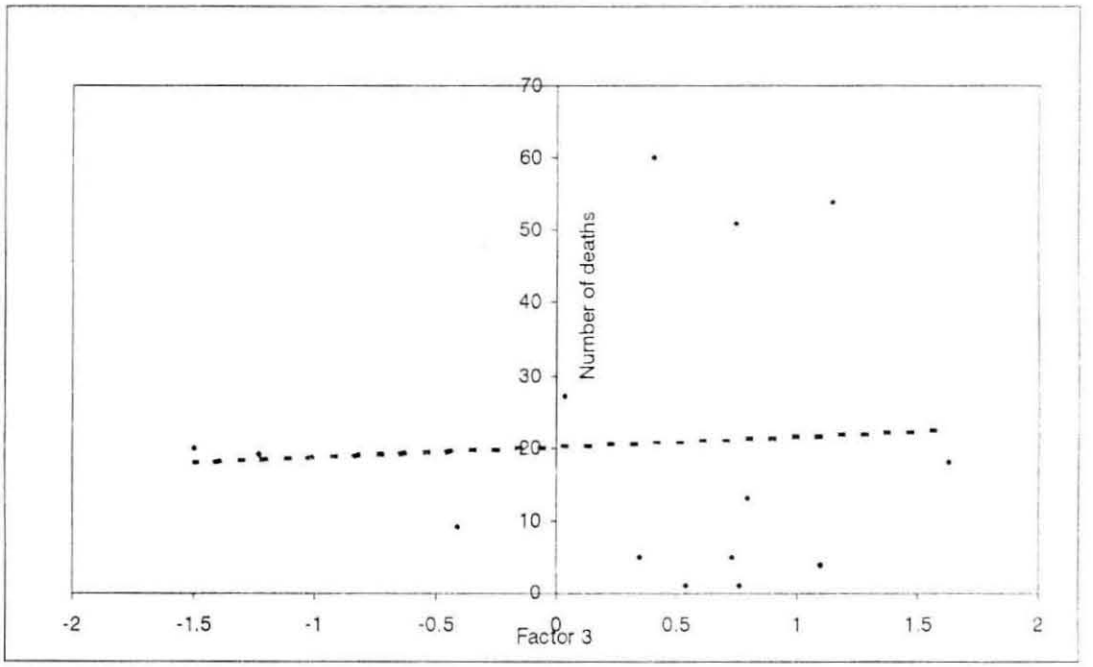


Fig. 12. Correlation of death trend with factor 3 of water chemistry index

Plate 1. *Chaetodon decussatus* showing visible external lesions

Plate 2. *Acanthurus bahianus* showing visible external leisions

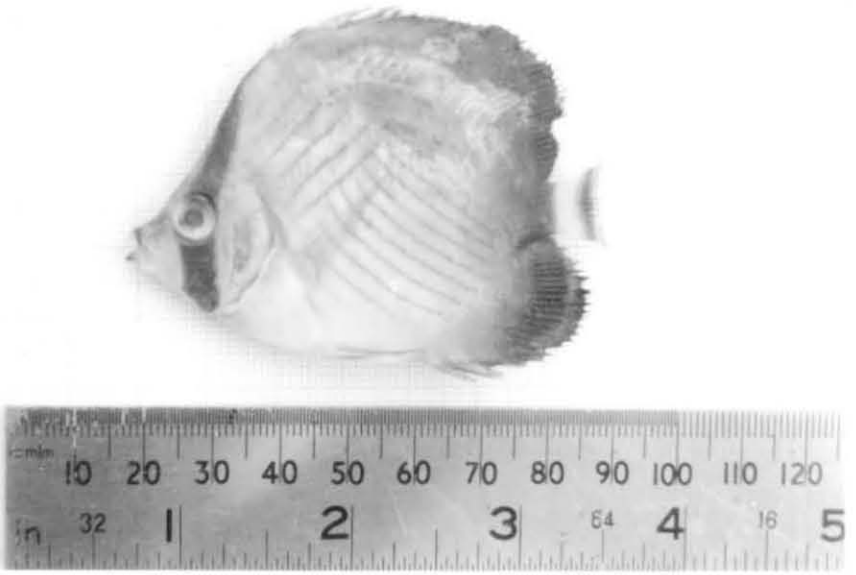
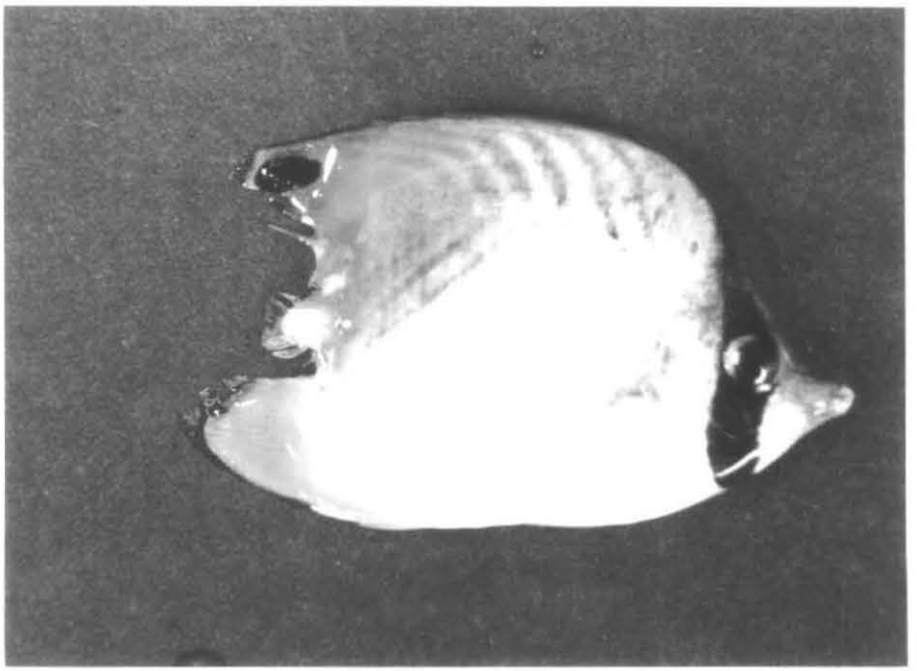
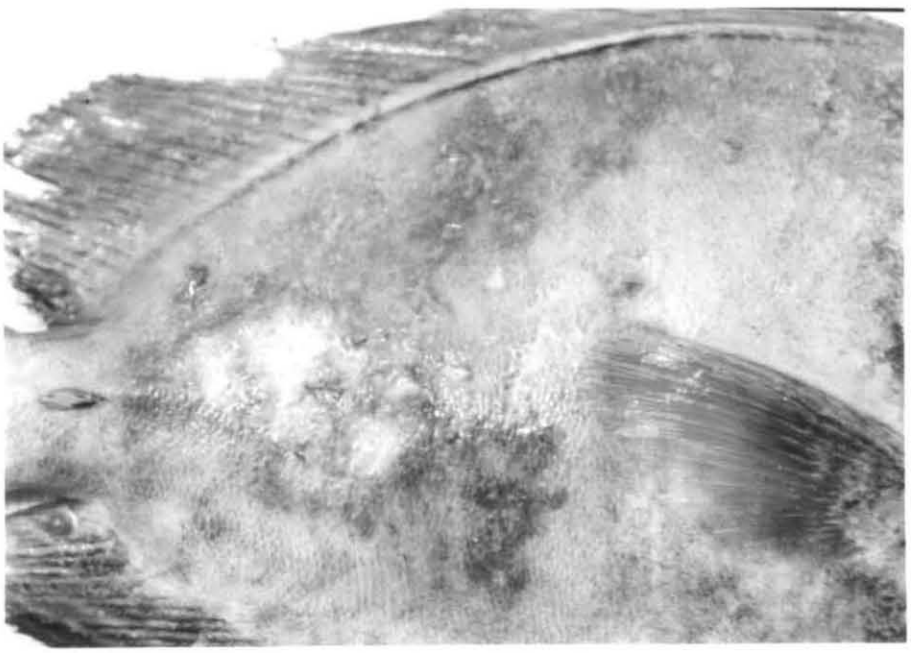

10 20 30 40 50 60 70 80 90 100 110 120
in 32 1 2 3 64 4 16 5

Plate 3. *Chaetodon auriga with* severe fin erosions

Plate 4. *Acanthurus bahianus* with ulcerations on lateral sides





Microbial load during this period was recorded as 29×10^4 which was higher than the normally recorded values.

Sudden occurrence of mortality was again noticed in February 1999 when damselfish died in large numbers comprising all age and size groups. Most of the dead fish did not show external signs other than wide-open mouth in the smaller fish. Some fish showed ulcerations on body. The microbial load ranged during this period ranged from 3 to 16×10^3 CFU/ml. Though the hydrological parameters were within the normal limits, the nitrate concentration was higher than the preceding and succeeding months, which ranged from 2.76 to 9.37µg at/l, while those of ammonia ranged from 0.95 to 2.05 µg at/l. It could be noted that the values of ammonia and microbial load were higher than those generally noticed.

Again in the first week of March 1999, sudden mortality was noticed in the aquarium mainly involving chaetodontids, callyodontids and clown fish. It was noticed that nitrate, nitrite and ammonia values (nitrate level was 0.9151µgat/l, nitrite, 0.1289µgat/l and ammonia 0.8125µgat/l) were comparatively lower than the values recorded during February.

Complete mortality of fish in one tank was noticed again in July 1999. All fish kept in the tank perished including siganids, acanthurids, and serranids of all size groups. It was observed that in the previous month higher microbial load of 200×10^2 was recorded. All the hydrological parameters were within permissible limits.

In August, all fish of one tank succumbed including groups such as acanthurids, callyodontids, chetodontids (showed slight fin rot symptoms), siganids and serranids. During this period the microbial load was extremely low in the aquarium. Similarly in October, immediately after tank resetting mortality was observed mostly involving monocanthids. Comparatively higher values of ammonia (0.82 to 1.11µg at/l) were noticed during this period in the aquarium.

DISCUSSION

The results of the present chapter indicated that the collective effects of hydrological parameters contributed to the onset of infections and mortality among the marine ornamental fish. It was stated by Plumb (1992), that proper management and environmental manipulation will help in controlling most of the diseases which are the results of stress, for which good quality water, free of contaminants and elevated levels of ammonia as well as other nitrogenous toxic metabolites and carbon dioxide concentrations are essential. In addition, supply of adequate dissolved oxygen, prevention of build up of organic matter and avoidance of temperature shock are critical.

A number of studies have demonstrated that various kinds of environmental stressors can have significant effects on the immune responses of fish (Sindermaan, 1990). The association between environmental stressors and outbreak of diseases in fish is probably linked to the immunosuppressive actions of stressors (Reddy and Leatherland, 1998). According to Wedemeyer *et al.* (1999) fish diseases do not occur in single caused event, but are the end results of interactions of the etiologic agent, the fish and the environment.

Any non-optimum environmental factor may act as stressor of fish, which if extreme enough or prolonged enough, may affect survival (Sindermann, 1990). The most widespread and significant factors are, stress from abnormal physical, chemical and biological conditions, including high temperatures, low dissolved oxygen, accumulation of metabolites, inadequate diets or overcrowding, which are capable of acting as predisposing factors of infectious diseases. Bry (1988) described deterioration of water quality as the most important factor leading to stress condition. In such circumstances, the opportunistic or facultative pathogens, often part of the normal microbial population, may emerge as significant pathogens of the captive fish, for which conditions in the marine aquarium are most favourable. In the Asia-Pacific region, ammonia and dissolved oxygen content in the rearing water have been

considered important factors influencing the health status of tropical fish (Braaten and Hektoen, 1991). In a study by Rigos *et al.* (1998), high mortalities of pathological origin were evident when juvenile common dentex (*Dentex dentex* L.) were exposed to stress situations and increased handling, and was found that the pathological conditions were associated with the relatively high susceptibility of common dentex to stress conditions. In another study by Diggles *et al.* (2000) it was seen that, infections by opportunistic bacteria in fish predisposed by a combination of adverse factors including an acute period of poor water quality along with inadequate diet led to mortality in hatchery reared turbot (*Colistium nudipinnis*) and brill (*C. guntheri*) in an experimental rearing facility in New Zealand.

It is well known that the natural habitats of tropical marine ornamental fish are specialised environments such as coral reef ecosystem, with clear water (Pichai Sonechaeng, 1988). The marine ornamentals are also known to live in close association with the invertebrates. However, when the organisms are kept in captivity, a mild fluctuation or variation in the water quality may lead to stressful conditions for the fishes. The aquarium where research was carried out was a well-maintained one, which simulated the natural conditions to the maximum level possible.

In the present study, the results revealed that important factors determining the general water quality included temperature, dissolved oxygen, pH, and nitrogen wastes. These parameters were considered to influence the general health of the fish under captivity. The following factors were studied in detail.

Temperature

The observations on atmospheric temperature and water temperature showed that the variations were kept minimum in the system. Changes in atmospheric temperatures were reported to be the main determinants of metabolic rate in fish. (Cossins, 1983). In the present study, however, the fishes were subjected to more or less constant atmospheric and

water temperatures with standard deviation of 1.44 and 1.18 respectively, which did not have any significant influence on fish health. However, declined values of temperature were one of the contributing factors of Factor 1 of water chemistry index (Table 3), but had no major role in mortality of ornamental fish as indicated in Fig. 10. This could have been due to the lower fluctuations in temperature. The absence of large fluctuations in water temperature could have been due to the continuous provision of artificial lighting in the tanks except during night.

Both high and low values of temperature can act as stress factors. Outbreaks of bacterial diseases have been associated with high temperatures of water (Ghittino and Prearo, 1992). Similarly, it was observed by Hrubec *et al* (1996) that lower temperatures below the optimum range were found to decrease the magnitude and delay the time of antibody response to *Aeromonas salmonicida*.

Pathogenicity studies with *A. salmonicida* indicated that higher water temperature not only increased the total mortality from furunculosis, but also shortened the time period between infection and death (Fryer and Pilcher, 1974). Though experiments proving this factor were not conducted in the present study, it is suspected that in combination with other factors, the temperature changes could have played a role in disease susceptibility, since temperature fluctuations contributed a major role in water chemistry. It was also suggested by Stoskopf (1993) that minor fluctuations in marine ecosystems could be sufficient to impose additional stress on fish maintained in otherwise marginal conditions, to precipitate the disease outbreaks a few days later. So, minor changes in temperature along with other environmental parameters could have led to stress to fish. It is also worth mentioning that as far as maintaining fish in captivity is concerned, compared to minimum or maximum limits of temperature, optimum temperatures are of interest (Wedemeyer *et al.*, 1999).

Even though, in the present study, bacterial diseases did not show any direct association with temperature prevailing in the system, the noteworthy

role played by temperature in water chemistry index, proved that it could be a determinant of stress.

Studies in the thirties and forties have confirmed the effects of temperature on immune response in fish (Allen and McDaniel, 1937; Bisset, 1948). It could be presumed that in the present study, which was conducted in a system, which simulated the natural habitat, the changes in water temperature, in addition to the fluctuations in the water temperature was capable of acting as one of the predisposing factors for disease occurrence.

pH

The pH values of aquarium had a very low standard deviation of 0.09, which indicated that the values fluctuated within a narrow range. However, the factor was capable of influencing the water chemistry and performance of the fish. Based on principal component analysis, the increased pH was estimated as the main component for factor 2 of water chemistry index (Table 3). Also, from Fig. 11, it was seen that higher occurrence of deaths coincided with lower values of factor 2. Thus it may be assumed that lower values of pH had a role in the occurrence of mortality. In the system which was studied, the values were lower than the usual pH of ocean water where it is normally above 8 at which it is strongly buffered and hence, acidity in salt-water fish culture rarely occur (Wedemeyer *et al.*, 1999). Nevertheless, the possibility of deaths due to acidity was not proved from the investigations.

According to Stoskopf (1993) the hydrogen ion concentration is vital for the survival of fish particularly in recirculating systems, where after a period of time, the buffering capacity of water is diminished, resulting in decreased pH due to constant addition of acidic waste products from the fishes and the biological filters. Changes in pH values were found to enhance microbial populations (Austin *et al.*, 1999) and enhance the progression of cutaneous ulcerative disease in sea mullet, *Mugil cephalus* as recorded by Virgona (1992). It can be assumed that the changes in values of pH to lower levels along with other stress factors like enhanced microbial load might have

lead to increased number of deaths in the aquarium, despite the fact that the variations in values were minimum.

Salinity

Salinity of the aquarium water also was not a factor leading to deterioration in water quality, but was a contributing factor of water chemistry index (Table 3). From Fig.11, it is clear that increased values of factor 3, for which salinity was an influencing factor corresponded with increase in deaths of the aquarium held fishes. Progression to later stages of cutaneous ulcerative disease in sea mullet, M. *cephalus* was reported to be due to changes in salinity along with other factors, as reported by Virgona (1992). Besides, studies by Bordas *et al.* (1996) has shown that kinetics of adhesion of *Vibrio* strains to skin mucus of gilt-head sea bream depended on several environmental factors including temperature and salinity. Another observation was that salinity showed a standard deviation of 1.38 and that the variations in the same did not correspond with expected seasonal changes, which could be due to the influence of other environmental factors and also due to the fact that seasonal fluctuations may not be a factor of concern in confined systems.

Dissolved oxygen

Oxygen content in aquarium showed a standard variation of 1.23 over the period of study with the maximum values recorded during the period from December to February (Fig. 4), which corresponds to transition period between post monsoon and pre monsoon. However, this factor did not show fluctuation due to seasonal changes, because continuous aeration was provided in the system. The increase in values of dissolved oxygen was an important deciding factor (Factor 2) of water chemistry (Table 3). Virgona (1992) observed that like salinity, dissolved oxygen was one of the factors, which favoured the progression of ulcerative disease in sea mullets. Studies by Bunch and Bejerano (1997) proved that low dissolved oxygen levels were capable of inducing mortality in hybrid tilapias due to *Streptococcus* infections. From

Fig.11, it is seen that lower values of factor 2 led to increased deaths in the aquarium, which corresponded, with these observations. Also, it is recorded by Stoskopf (1993) that dissolved oxygen levels greater than 7 ppm is preferable. Values greater than 7 were recorded in the system only in samples collected during the months of December to February and June to August, which correspond to transition period and monsoon despite the fact that the seasonal variations in water quality parameters may not have much impact on fishes reared under captivity.

Excess dissolved gases in water leads to gas bubble disease, which comprise a variable range of lesions (Speare, 1998), but this condition was insignificant in the aquarium under study, and saturation due to dissolved oxygen was totally absent.

Ammonia

The values of nitrogen wastes in the from of ammonia, nitrate and nitrite were kept low in aquarium water. For ammonia, the values were always less than the toxic limits and the maximum recorded value was 1.7 µg at per litre.

Ammonia content in the aquarium water was maintained low in the aquarium by means of the biological filter. However, fluctuations in ammonia content were found to coincide with variations in nitrate and nitrite content and a significant positive correlation was found to exist between ammonia content and nitrite content (Table 2). Higher values of these parameters were observed in the periods from September to November and June to August corresponding to post monsoon and monsoon seasons (Table 1). This could be due to the presence of increased amount of nutrients in the seawater during these periods. Higher values of these parameters usually lead to enhanced microbial load (Table 1), which in turn results in increased susceptibility to disease occurrence. This condition could have been the cause of increased number of deaths in the aquarium, which is evident from Fig. 9, 10 and 11. A significant positive correlation was also found to exist between ammonia content and factor 1 of

water chemistry index. Fish differ in their susceptibility to ammonia. For many fishes, 0.02 mg NH_3/l is about the maximum, which can be tolerated chronically without adverse effects (Wedemeyer *et al.*, 1999).

Ammonia concentrations at levels higher than 0.05 mg/ l is also thought to be a risk factor of susceptibility to *Aeromonas* species (Ortega, 1996) in rainbow trout hatcheries and in recirculating culture systems (Noble and Summerfelt, 1996), in addition to causing fin erosions in rainbow trout hatcheries (Bosakowski and Wagner, 1994). According to Cordova *et al.* (1996), most of the lesions observed in fish in confinement are due to excess levels of ammonia in the system. High levels of ammonia is detected to be leading to stress in sea-cage reared sea bass and sea bream as observed by Papoutsoglou *et al.* (1996). Branchial lesions from ammonia exposure have been reported (Smith and Piper, 1975, Smart, 1976, and Thurston *et al.*, 1984), which according to Meade (1985) have been linked to unionised ammonia.

In general, the ammonia toxicity is related to the pH prevailing in the water (Wedemeyer *et al.*, 1999). Though the ammonia content was within the allowable limits in the present study, mortality of fish were recorded whenever increase in trend in ammonia was also noted. As already stated, this could have been due to multifactorial reasons.

Nitrite

Nitrite content in aquarium was an important determinant factor of water chemistry as evident from the results of factor analysis (Table 3). This factor is significant in susceptibility to many bacterial disease outbreaks. According to Hanson and Grizzle (1985), channel catfish are predisposed to bacterial diseases during chronic exposure to high nitrite levels. Although no association was found between the nitrite content and occurrence of fish deaths, from the present study, it is presumed that, nitrite could be a deciding factor at its extreme levels. In the present investigations nitrite levels were never more than 0.52 µg at/L and the standard deviation was 0.39, which indicate that, the variation in values were minimum.

High levels of nitrite are reported to be the reason for mortality in recirculating systems for rainbow trout cultures (Noble and Summerfelt, 1996; Noble, 1996). Toxic concentration of nitrite levels from 1-5 ppm was observed in zebra fish rearing facilities, due to absence of colonising nitrifying bacteria in the biological filter, causing build up of nitrite in the system, which along with overcrowding, led to susceptibility to motile aeromonad septicemia as recorded by Pullium *et al.* (1999). The aquarium under evaluation had well functioning biological filter, which was capable of eliminating high levels of nitrogen wastes.

Nitrate

Nitrate content in the aquarium was one of the decisive factors of water chemistry (Table 3) and affected factor 1 of principal components of water chemistry. It showed wide fluctuations in values over the period of study, with a standard deviation of 6.34. Higher values were observed in samples collected during periods corresponding to monsoon and post monsoon period (June to September), which could be due to the increased load of nutrients in water. Elevated levels of nitrate were found to reduce the antibody response in sunshine bass (Hrubec *et al.*, 1996). The studies of Bunch and Bejerano (1997) in tilapia hybrids indicated that high nitrate concentration led to susceptibility of the fish to streptococcal infection. Pullium *et al.* (1999) also indicated that, by reducing the nitrate level in the rearing water, the septicaemic condition and susceptibility to aeromonad infection could be minimised. Like ammonia, nitrate also could be associated with the incapability of hemoglobin to combine oxygen leading to hypoxic conditions. However, there was no direct association of increased nitrate content with deaths in aquarium.

In general, it was noted that compared to other parameters of water quality, ammonia, nitrate and nitrite content acted as primary predisposing factors of disease occurrence. For marine tropical fish under captivity, these factors are of excessive significance. According to Hiatt (1988), the increased levels of factors such as ammonia, nitrite and nitrate in aquaria enhance the chance of outbreak of diseases.

In samples collected during periods corresponding to the premonsoon period, (March to May) the salinity and temperature were higher with a concomitant increase in ammonia level during which period high mortality was recorded in the present study (Table1, Fig. 4, Fig. 8). The pomacentrids suffered maximum mortality during this period. A similar trend was also recorded during the monsoon period (June to August), during which time the nitrate values were also high.

Microbial load

Although there is no specific correlation existing between microbial load in the aquarium and the mortality of fish, generally it was seen that the bacterial load was enhanced during June to August, which correspond to the monsoon period. This increase in microbial load could be due to the enhanced nitrate levels, as noticed from Table 1 and Fig. 7. These patterns also suggest that the prevalence of bacterial infection and consequent mortality need not be associated with the background microbial load since there was no significant correlation between microbial load and other environment factors or with total number of deaths.

Reports of multiplication of pathogenic microflora under poor water quality conditions have been documented (Noble, 1996). It can be noted from Table 1 that average values for the parameters of water quality including nitrate, nitrate and nitrite as well as microbial load were higher during June to September which corresponds to the monsoon and post monsoon period, which indicated the probability of increased microbial load resulting from deterioration of water quality.

Occurrence of mass mortality

Based on the observations when sudden mortality occurred in the aquarium,it may be inferred that mortality occurred in association with decrease in values of dissolved oxygen and with both high and low values of microbial load. From the values of ammonia (0.95 to 2.05 µg at/L) and microbial load,

which showed a high value of 160×10^3 during first half of February 1999, it could be noted that the enhanced values could have contributed to the mass mortality.

It was also noticed that sudden variations in values of nitrate, nitrite and ammonia led to spontaneous occurrence of mortality. For example, during the period March 1999 low values of these factors were recorded and the ensuing lower nutrient status might have become the cause of mortality.

Another observation was that following resetting of tanks when the filter was undergoing colonisation of bacteria, the fishes stocked were more vulnerable to diseases, since the metabolites were not transformed to less harmful forms. The resetting of tank following mortality in the previous month, which did not provide enough time for proper colonisation of bacteria in the filter led to decline in microbial load and resulted mortality in the months of August and October, 1999. Absence of colonising nitrifying bacteria in the biological filter, causing build up of nitrite was recorded by Pullium *et al.* (1999), which along with overcrowding led to susceptibility to motile aeromonad septicemia.

It can be concluded from the present study that the marine aquarium with the filtration system was maintained well and that the slight fluctuation in the water chemistry did not affect the microbial load in the system. However, the general death pattern of fish in the aquarium showed that, mortality occurred with slight increase in levels of ammonia, nitrate and nitrite, which can be considered as the outcome of collective effect of environmental and biological factors.

Combinations of environmental and host factors are cited as causing similar range of disease outbreaks in intensively reared species (Mellergaard and Dalsgaard, 1987). The way in which various factors affecting the host, pathogen and environment interact, decides the occurrence of diseases. In the present study conducted in the marine aquarium also, the interactions of various factors can be considered as the reasons for the onset of mortality among fishes.

CHAPTER 2

ISOLATION AND CHARACTERISATION OF POTENTIAL BACTERIAL PATHOGENS

Bacteria associated with mariculture can be classified as 1. Primary pathogens 2.Secondary invaders often with proteolytic activities that may be pathogenic for host with pre-existing infections 3. Proteolytic heterotrophs, which invade dying animals, and which if cultured and injected experimentally in massive quantities, may kill some experimental hosts and 4. Normal microflora, which may occur on body surfaces of the host but are not pathogenic (Sindermann, 1990). Conroy in 1984 attempted to classify the bacterial groups in a useful way, involving most of the important bacterial pathogens of marine fish which were, Gram negative organisms, acid-fast bacteria, Gram positive pathogens, anaerobic bacteria and Myxobacteria.

A critical problem in the study of bacterial pathogens of the fish is the correct identification of the infectious agent. To facilitate the precise identification of these organisms, which may be occasionally involved in disease processes, the usual practice is to perform biochemical tests and thus identify the isolates by reactions shown by them in standard tests, or to go for techniques of molecular biology or serology which is carried out in order to find out, to which genus, species or subspecies it belongs.

Identification of bacterial isolates based on biochemical tests is a classic method followed by microbiologists. Because of many of the obscurities involved in bacterial taxonomy, biochemical characterisation also can be incomplete or confusing (Austin, 1982; Sindermann, 1990). Attempts to classify the major groups have been done in the past especially for vibrios (Shewan *et al.*, 1960; Colwell and Liston, 1960; Colwell and Grimes, 1984).

In the present chapter, morphological characters such as colour and appearance were noted along with their staining properties as primary

criteria for identification. Following this, a range of biochemical tests were done to categorise the Gram negative bacteria isolated from the infected marine ornamental fish using standard biochemical tests.

Important Taxonomic Groups of Bacteria Encountered in Marine Fish Rearing Facilities

The normal flora of the marine fish reflects the environment in which they live. The diverse array of bacteria inhabiting the surface and gills are reflections of the range of bacteria normally present in water (Colwell, 1962; Austin, 1982). Therefore, in order to understand the role of a microorganism in an incidence of infection, or disease outbreak, the detailed information about the seasonal distribution, types (qualitative data) and numbers (quantitative distribution) of microbes is essential.

Majority of the bacterial flora involved in marine and aquatic environment comprise Gram negative bacteria (Gratzek *et al.*, 1978) and they are asporogenous rods motile by polar flagella (Pfister and Burkholder, 1965). As observed by Shotts *et al.* (1976), bacterial flora of shipping waters of ornamental fishes is found to show a marked identity in different segments of study, where again the predominant flora was reported to belong to Gram negative rods like *Pseudomonas*, *Aeromonas hydrophila* complex, along with *Enterobacter* spp., *Escherichia* spp., *Flavobacterium* and *Proteus vulgaris*.

The numbers of aerobic heterotrophic bacteria on the fish equate with that of the environment. (Austin and Austin, 1987). Quantitative studies on the bacteria revealed that fish skin harbour 10^2 to 10^4 bacteria per cm^2 (Horsely, 1973; Gillespie and Macrae, 1975) whereas gills harbour up to 10^6 numbers per gram of tissue. Electron microscopic studies have proved that most of the organisms are loosely attached to surface of fish (Austin and Austin, 1987). Bacterial numbers appeared to be high in the intestinal tract up to 10^8 per gram (Yoshimizu *et al.*, 1976) indicating the presence of favourable ecological niches for microorganisms.

The surface of marine fishes are populated by a wide variety of bacterial genera, typical of seawater including *Acinetobacter calcoaceticus*, *Alcaligenes faecalis*, *Bacillus* spp., *Caulobacter*, coryneformes, *Cytohaga/Flexibacter*, *Escherichia coli*, *Hyphomicrobium vulgare*, *Lucibacterium harveyi*, *Photobacterium* spp., *Pseudomonas* spp. and *Vibrio* spp. (Austin, 1983). The gills are reported to be inhabited by populations of *Achromobacter*, *Alcaligenes*, *Bacillus*, *Flavobacterium* and *Micrococcus* (Shewan, 1961). From the guts of marine fish, the predominant taxa reported are *Vibrio* (Colwell, 1962; Simidu and Kaneko, 1969; Sakata *et al.*, 1980), *Pseudomonas* (Liston, 1957), and *Aeromonas* (Ugajin, 1979). Liston (1957) had suggested that the conditions in the gut are favorable for vibrios in particular, to survive, which has been proved by subsequent workers (Newman *et al.*, 1972; Yoshimizu and Kimura, 1976). Gut group vibrios are isolated commonly from various marine fish including skate, lemon sole (Liston, 1957), red sea bream (Muroga *et al.*, 1987) Salmonids (Yoshimizu and Kimura, 1976) yellow tail (Sakata *et al.*, 1978) and puffer fish (Sugita *et al.*, 1987). It was summarised by Austin and Austin (1989) that whereas body surface maintains a microflora containing *Acinetobacter*, *Flavobacterium /Cytophaga*, *Moraxella* and *Pseudomonas*, representatives of family Vibrionaceae occur in the intestinal tract.

Despite the rapid development in mariculture, microbiological aspect of marine fish farming and more importantly, the involvement of bacteria in fish diseases received little attention in the early literature (Austin, 1982). In spite of the industry being a well-established one, bacterial diseases outbreaks were not studied in detail. As stated elsewhere in the thesis, very little amount of work has been done with respect to the bacterial infections of marine ornamental fish. Another factor of concern is the presence of bacteria of zoonotic importance encountered in the marine aquarium. The chance of occurrence of human pathogens in the marine aquarium is discussed by Mualu and Ijumba (1982) and Vandepitte *et al.* (1983). The origin of such infections, from aquarium or aquarium fish is of significance and has to be avoided at any cost.

It is advisable to have a better understanding of the systematics of bacteria, so as to have a complete knowledge of the marine micro flora, which may have roles in pathological conditions. Biochemical methods are commonly followed for routine purposes of identification and the results are interpreted based on the characteristics shown by the isolates when standard biochemical tests are conducted. However, marine bacterial identification presents problems, since most of the conventional schemes help in identifying up to genus level alone (Austin *et al.*, 1979b). Isolates classified within the same group on the basis of some characteristics may also differ in many others. Identification of *Vibrio* species is a good example for such difficulties (Alsina and Blanch, 1994).

Based on the characteristics of typical organisms associated with lesions, it is possible to identify pathogens. A schematic outline for presumptive identification of bacterial diseases of fish given by Bullock (1961) and Shotts and Bullock (1975) is ideal as far as some pathogens are concerned. However, identification up to species level of important bacteria from marine environment is a tough task and hardly any single scheme offers possibilities for precise taxonomic classifications.

In 1960, Shewan *et al.* presented a scheme for identification of certain genera of aerobic heterotrophic Gram negative bacteria, especially the Pseudomonadaceae. This scheme which has been in wide use since then, helped to divide the aerobic heterotrophic Gram negative rod shaped bacteria into four groups on the basis of motility, flagella, oxidase reaction and pigment production. They were further subdivided into *Pseudomonas*, *Aeromonas* and *Vibrio* and later to *Alcaligenes* and *Achromobacter*.

In early descriptions many disease conditions involved the genus *Pseudomonas*, but with increased knowledge of bacterial taxonomy and improved methods of identifications many are assigned to other genera. The developments in taxonomy and identification since 1960 are the result of

applications of new approaches and techniques involving numerical analysis, molecular biology and genetic studies, apart from intermediary metabolic processes.

Numerical taxonomy helps in classifying related organisms into clusters, especially in the case of large populations, as in the sea (Pfister and Burckholder, 1965; Baumann *et al.*, 1972). But identification of specific groups again presents problems, since they may differ in many characters other than the key characters.

Characteristics of Major Disease Causing Bacteria in the Marine Environment

Worldwide, approximately thirty-five species of bacteria belonging to twenty-one genera have been isolated from or associated with diseased fish. Of these, twelve species of bacteria from nine genera are considered major pathogens of fish and five are considered obligatory pathogens. As indicated earlier, significant presence of human pathogens cannot be ruled out, since nine species belonging to seven genera are involved in bacterial diseases of humans (Sanders and Fryer, 1988). It was recorded by Oppenheimer and Kesteven (1953) that, of all the external lesions on fish reared in salt-water aquaria, bacterial fin rot and tail rot are most common. The commonest of bacterial infections are best characterised as haemorrhagic septicaemia and were identified to be due to infections caused by *Vibrio-Pseudomonas -Aeromonas* complex of bacteria (Sindermann, 1990).

Gram negative bacteria

Vibrios are ubiquitous in marine and estuarine waters and are parts of normal flora of intestine of many fishes. Infections due to *Vibrio* spp. were documented since the 19th century onwards and are significant causes of mortality in marine fish. More than 40 species were reported from wild and

cultured fish (Anderson and Conroy, 1970) of which V. *anguillarum, V. ordalli, V. salmonicida, V. alginolyticus V. vulificus, V. harveyi and V. damsela*, were reported as marine fish or shellfish pathogens (Hjeltnes and Roberts, 1993). Other genera belonging to the same family include *Aeromonas*, *Pleisiomonas*, *Photobacterium* etc. that are all facultatively anaerobic, generally oxidase positive, polar flagellate and Gram negative rods.

Extensive studies have been conducted regarding the family and group, within these, and are now fairly well established. Simplest keys as that of Bain and Shewan (1968) to complicated ones based on DNA base ratio etc were also used.

Opportunistic bacterial pathogens have been identified as being involved in fish mortalities when present in high enough numbers and/or when the fish's defense mechanism is weakened (Hispano *et al.*, 1997). Several Vibrio species have been identified in this context. The DNA hybridisation tests indicate high percentage of binding between species. The molecular percentage G-C ratio is distinct when compared to *Aeromonas*. Other tests of significance in identifying them are decarboxylase test, sensitivity to Vibriostatic agent 0/129, Novobiocin etc. Studies have also been conducted in order to classify the relationship between other genera within the family, including characteristics such as luminescence, flagellation etc.

V. anguillarum is a well-known fish pathogen causing vibriosis in many fishes all over the world, including cultured ayu in Japan (Anderson and Conroy, 1970; Muroga and Egusa, 1967; Kusuda *et al.*, 1979). The disease occurrence characteristics due to *Vibrio* species vary and a clear seasonal pattern of occurrence is indicated, which can be due to variations in optimum temperature conditions for growth.

The aeromonads are isolated from waters of widely varying physicochemical limits in both oligosaprobic and polysaprobic systems (Doukas

et al., 1998). They can be isolated from coastal and brackish waters where salinity is low and intense degradation of high molecular compounds occur (Kaper *et al.*, 1981; Boira, 1996). Infections in marine fishes like Atlantic salmon (Candan *et al.*, 1995) and in sea bream (Doukas *et al.*,1998) were also reported recently. Overcrowding is considered as a predisposing factor for aeromonad infection.

Aeromonas can be distinguished from other related genera because of its lower requirement of sodium chloride and different molecular percentage G-C ratio. The species *A. hydrophila* has been mostly isolated from fresh water and brackish water environments and is the causative agent of motile aeromonad septicemia in many aquatic animals. It is ubiquitous and opportunistic and forms a part of normal micro flora of fish (Hazen *et al.*, 1974; Shotts *et al.*, 1980; Ansary *et al.*, 1992) and causes disease under stress conditions (Kou, 1973; Boulanger *et al.*, 1977; De Figueiredo and Plumb, 1977). *Aeromonas salmonicida* is a true fish pathogen and cause furunculosis among salmonids. Typical and atypical isolates of *A. salmonicida* were reported which were involved in disease symptoms.

As a predominant genus, the pseudomonads are identified based on the important biochemical reaction viz. metabolism in oxidation fermentation medium. They are divided into four groups based on the type of metabolism shown (Simidu and Aiso, 1962). A closely related genus, *Alteromonas* is differentiated based on G-C ratio alone. The pseudomonads are straight to slightly curved Gram negative rods and are motile by polar flagella. The genus *Pseudomonas* is frequently associated with bacterial hemorrhagic septicaemia of aquarium fish (Bullock and McLaughlin, 1970) and twenty seven recognised spp. are reported to cause diseases in fish (Sanders and Fryer, 1988) and are frequently isolated from fish (Cahill, 1990a) and are found on eggs (Bell *et al.*, 1971) skin and gills (Colwell 1962; Horsely, 1973) and intestine (Austin and Al-Zaharani, 1988; Trust and Sparrow, 1974)).

The pseudomonads are generally considered falcultatively pathogenic genera although one species, *P. anguilliseptica* is reported as a primary pathogen (Wakabayashi and Egusa, 1972) and is the causative agent for a serious red spot disease (sekiten-byo) of cultured eels in Japan and Europe. Other species, *P. flourescens* (Bullock and Mc Laughlin, 1970) and *P. chloraphis* are reported to cause disease in salmonids, though mortality is observed in cases involving skin injury to the fish. A wide variety of hosts are reported for *P. fluorescens* (Sanders and Fryer, 1988). The related genus *Alteromonas* is confined mostly to marine habitat. *A. putrefaciens* is associated with fish spoilage and is reclassified as *Shewanella*. *Alteromonas piscicida* is another known species (Inglis and Hendrie, 1993).

The Pasteurellaceae family includes the genus *Pasteurella* which are Gram negative small coccoid rods and separation from species of other genera like *Haemophilus* and *Actinobacillus* is difficult. The useful characteristics in identifying this genus are positive oxidase reaction, alkaline phosphatase reactions and sensitivity to benzyl penicillin (Sanders and Fryer, 1988). *Pasteurella piscicida* is the identified pathogen of marine and euryhaline fish and was found to be the cause of fish kills due to an epizootic in which perches and stripped bass were killed causing a considerable decline in population (Janssen and Surgalla, 1968). Pasteurellosis was also reported as the cause of mortality due to a symptom designated as pseudotuberculosis (Sanders and Fryer, 1988).

Flavobacterium and yellow-pigmented rods are Gram negative bacteria motile by peritrichous flagella or non motile without any gliding/swarming movement. These are differentiated from genus *Cytophaga/Felxibacter* by types of motion and by DNA base ratio. They are usually associated with bacterial gill disease (BGD) outbreaks, (Wakabayashi *et al.*, 1980) which are associated with stress factors like crowding, low dissolved oxygen, and high ammonia concentration that are commonly observed in aquaculture facilities. The salmonids as well as other fishes are hosts. Accurate

taxonomic identification of yellow-pigmented bacteria is found to be unsatisfactory (Nogi *et al.*, 1997).

The genera, *Flexibacter* and *Cytophaga* comprise Gram negative weakly retractile flexible rods showing gliding motility on solid surfaces, the motility being the single most important character. The family Cytophagaceae includes other genera also, and can be separated based on cellular characters and biochemical tests. Members inhabit soil as well as freshwater and marine habitat. The species *F. maritimus* requires salt for growth and was isolated from external lesions of marine fish (Wakabayashi *et al.*, 1986). It was found to be the cause for an erosive skin disease in Japanese aquaculture species like red sea bream and flounder (Baxa *et al.*, 1986). Sole and turbot have been found to be susceptible (Alsina and Blanch, 1993; Bernardet *et al.*, 1990) and is an identified pathogen for salmonids.

The Enterobacteriaceae family includes pathogens of species like *Edwardsiella, Yersinia* and many other potential isolates like *Hafnia*, *Serratia* and, *Escherischia* from fish. Many species belonging to these genera are found in the alimentary tract of salmonids and can be considered as opportunistic pathogens.

Edwardsiella tarda is a species, which has gained public health significance recently (Sindermann, 1990; Vandepitte *et al.*, 1983) because of its obvious role in human gastro enteritis. It is associated with many aquatic vertebrates and there have been many reports on the isolation of this species and its pathogenic role in fish. Clinical signs due to *Edwardsiella* infection are seen in Tilapia, stripped bass and eels. *Edwardsiella* septicaemia in most fish species is favored by high water temperature and increased presence of organic matter.

Another important genus considered as pathogen of fish is *Yersinia*, consisting of seven species. *Y. ruckeri* is pathogenic to salmonids

causing enteric red mouth disease. The mortality level is found to be increasing with the presence of stress factors like overcrowding, low dissolved oxygen, temperature, immunity and resistance of the population. It differs from genus *Edwardsiella* by certain biochemical features like citrate utilisation, gelatin liquefaction and trehalose utilisation. (Sanders and Fryer, 1988).

Hafnia is often isolated from fish (Sanders and Fryer, 1988), and is reported to be the cause for epizootic hemorrhagic septicaemia and kidney pathology in salmonids. It is considered as an opportunistic pathogen in association with predisposing factors although, its status as a primary pathogen is being questioned (Sakazaki and Tamura, 1992).

The genus *Serratia* of the Enterobacteriaceae family comprise organisms which have potential for pathogenicity, including *Serratia liquefaciens*, *Serratia marcescens* and *Serratia plymuthica* which have been associated with bacterial septicaemia and mortalities in salmonids and are recognised as emerging pathogens (Austin and Gibb, 1993), though these species were considered opportunistic pathogens. There have been reports of *S. liquefaciens* as a pathogen of Atlantic salmon (McIntosh and Austin, 1990). Austin and Austin (1989) recorded that *S. liquefaciens* is emerging as fish pathogen in its own weight. Another species, *S. plymuthica* is associated with pollution and leads to surface lesions (Austin *et al.*, 1999).

Acid fast bacteria

The prevalence of bacterial diseases due to acid fast bacteria including *Mycobacterium* and *Nocardia* spp. which are characterised by a chronic debilitating disease syndrome of kidney, liver, spleen and gonad pathology are common in marine euryhaline fishes (Sindermann, 1990) and leads to mortality after several months. In the marine aquaria there are reports of rapid courses of acute infections occurring at an epizootic level (Giavenni *et al.*, 1980).

Fish tuberculosis (mycobacteriosis) has been reported from a number of commercial marine fish including halibut, cod and plaice (Sutherland 1922; Johnstone, 1927). Three species viz. *M. marinum, M. fortuitum* and *M. cholerae* have been found pathogenic on fish. More work on pathogenicity and host ranges has been done in 1950's and was found to cause serious diseases in Pacific salmonids which could have possibly been due to early exposure to bacilli in hatchery diets, which included carcasses of tuberculous fish (Earp *et al.* (1953) Wood and Ordal (1958), Ross *et al.* (1959), Ross (1960). Acid fast bacterial infection was noticed in cultured striped bass on the Pacific coast of the U.S. (Hedrick *et al.*,1987).

Two species of *Nocardia* were isolated from fish, *N. asteroida* and *N. kampachi* the latter being identified as a pathogen of yellow tail in Japan (Kusuda and Taki, 1973) causing systemic infections. The anadromous salmonids were reported to be infected by *Nocardia* species (Wolke and Meade, 1974).

Gram positive bacteria

A number of reports describe infections by Gram positive bacteria in cultured species of fishes. The important pathogens include, *Renibacterium salmoninarum* (causative agent of bacterial kidney disease), which infect fish and cause mortality in salt water (Bell, 1961; Ellis *et al.*, 1978). The genus, *Streptococcus* has been found to be causing problems in yellow tail culture in Japan. Infection is most common in first and second year of life and was found temperature dependent (Sindermann, 1990).

Sample Collection

In order to isolate the potential pathogenic bacteria, recently dead or moribund fishes were collected from the marine aquarium, CMFRI, Vizhinjam in sterile polyethene packets and brought to the laboratory for further analysis as and when bacterial infection was suspected.

Isolation of Pathogenic Bacteria

Using a sterile loop, inoculum from the lesions of fish, like ulcerations, erosions, or from the internal organs such as kidney, intestine etc. was streaked on a suitable medium prepared in plates. Sea Water Agar (SWA, composition: peptone - 1%, agar-2%, ferric phosphate – a pinch, aged sea water, pH-7.2, 15lbs; 20 minutes) and a specific medium, TCBS agar (Thiosulphate Citrate Bile Salt sucrose medium) (HiMedia) were used.

Inoculation was done by the streak plating method. After overnight incubation period, purified individual isolates were obtained. Alternatively, organs or parts of body showing lesions were inoculated in peptone broth and incubated overnight, after which a loopful of culture was streaked on agar plate or 0.1 ml from the broth was spread/pour plated and incubated. Isolated colonies were stored in agar slants for further biochemical characterisation. Cultures obtained were made free of contamination at frequent intervals by streak plating and subculturing.

Biochemical characterisation of pathogenic isolates

The following standard morphological characterisation criteria and biochemical tests were conducted to identify the important bacterial isolates.

Gram staining

Two dyes viz. crystal violet and saffranin (counter stain) were used to differentiate the bacteria based on staining properties of bacterial cell wall (Hucker and Conn, 1923; Lillie, 1928), which helps to categorise the bacteria into Gram positive (purple stained) or Gram negative (pink stained).

Motility test

A loopful of young peptone broth culture was placed on a cover slip on which a cavity slide, with its concave portion facing the drop was inverted (hanging drop method, as given by Collins and Lyne in 1976). The edges were sealed with vaseline and observed under microscope for motility of bacterial cells.

Cytochrome oxidase test

Whatman filter paper no.1 was wetted with a few drops of 1% solution of N'N'N' tetramethyl diamine dihydrochloride. The test organism was streaked on the filter paper with a loop. Immediate appearance of a purple colour indicated a positive reaction (Kovacs, 1956).

Hugh and Leifson's (H&L) oxidation fermentation test

H&L medium (HiMedia) was prepared and dispensed in tubes and were stab inoculated. Duplicate tubes were also inoculated and sealed with sterile liquid paraffin. After incubation period, the tubes were examined for acid production, which was indicated by the colour change of the medium (Hugh and Leifson, 1953).

Kovac's oxidase test

Following incubation in peptone broth, 2ml of Kovac's oxidase reagent (composition: Amyl alcohol-150 ml, p-dimethyl amino benzaldehyde-10g, conc. HCl-50 ml (aldehyde is dissolved in ethanol) was added to the

culture. The development of a pink colour indicated positive reaction (Kovacs, 1928).

Hydrogen sulfide production

A filter paper strip previously impregnated with saturated lead acetate solution was kept hanging into the peptone water culture of test organism, after inoculation and incubated. Darkening of the filter paper strip indicated the production of hydrogen sulphide by the bacteria (Barrow and Feltham, 1993).

Starch hydrolysis

Starch medium (composition: soluble starch –0.2%, beef extract-0.3%, agar-1.5%, distilled water, pH-7.2, 10lbs: 30 minutes) was prepared and plated followed by inoculation, by streaking with the culture. Following incubation at 37^{0}C for 48 hours, plates were flooded with Lugol's iodine solution. Zones of clearing around the culture indicated starch hydrolysis by the bacteria (Barrow and Feltham, 1993).

Gelatin Liquefaction

Gelatin medium (composition: peptone-0.2%, beef extract-0.2%, gelatin-12%, distilled water, pH-7.2) was prepared and dispensed in tubes and sterilised by tyndallisation. The medium was dispensed in tubes and stab inoculated and incubated at 37^0 C for 48 hrs (Barrow and Feltham, 1993). The tubes were examined for liquefaction of the medium after the incubation period.

Sugar fermentation

1.0% of the test sugar was added to peptone broth. After placing Durham's tubes in inverted position inside the tubes, medium was dispensed into the tubes without formation of air bubbles and sterilised at 115 ^{0} C for 10 minutes. The medium was inoculated with the test culture. Acid production and

gas bubble formation in the Durham's tubes were recorded after the incubation period of 24 hours at 37^{0}C.

Sodium chloride tolerance

Varying concentrations of sodium chloride (at 0%, 5% and 7% levels) were added to peptone broth medium, which were inoculated with test organism and checked for growth indicated by turbidity after incubation at 37^{0}C for 48hrs.

Methyl red test

MR-VP (Methyl red-Voges Proskaur) medium (HiMedia) was prepared and dispensed in tubes. The test organism was inoculated and incubated at 37^0 C for 5 days. Following incubation period, five drops of 0.001% of methyl red was added and colour change to pink was recorded as positive (Mackie and McCartney, 1953).

Voges-Proskaur Test

The glucose phosphate medium (MR-VP medium) was inoculated and incubated at 30^0 C for five days. After the incubation period, 3 ml of Alcoholic alpha-naphthol and 1 ml of 40% sodium hydroxide solution was added. A deep pink colour developing within 15 minutes to one hour was recorded as the positive result (Mackie and McCartney, 1953).

Amino acid decarboxylase tests

Falkow's medium (composition: Peptone-0.5%, Yeast extract-0.3%, Glucose-0.1%, Bromocresol purple solution-0.2% aqueous solution-10ml, 15lbs; 20 minutes) was prepared and 0.5% of the amino acid (arginine, lysine and ornithine) was added and dispensed into tubes and sterilised by steaming. Test and control tubes were inoculated and incubated at 37^0 C for 24 hrs or for longer if negative. Colour change to purple was recorded as positive (Collins and Lyne, 1976, Barrow and Feltham, 1993).

Urease test

The Christenson's urease medium was prepared (composition: peptone-0.1%, sodium chloride-0.5%, potassium dihydrogen phosphate-0.2%, agar-2%, phenol red-0.0012g, sterile glucose 10% solution-1ml, sterile urea, 20% solution-10ml, pH-6.8-6.9; 121^{0}C, 30minutes) and inoculated. Following incubation at 37^{0}C for 48 hours, the slopes were examined for change of colour to pink which represented positive reaction (Barrow and Feltham, 1993).

Citrate utilisation test

Simon's citrate agar medium (HiMedia) was prepared and dispensed into tubes to form deep slopes. The medium was heavily inoculated with test culture and incubated at 37 ^{0} C for 48 hrs. Colour change to pink was recorded as positive test.

ONPG test

To sterile phosphate buffer, 0.6% ONPG (*O*-nitro-phenyl-D-galactopyranoside) solution was added aseptically (3:1 ratio). The medium was checked for sterility for 24 hours and then inoculated. Following incubation at 37 ^{0} C for two days, colour change to yellow was recorded as positive.

0/129 sensitivity test

0/129 (2, 4- diamino 6, 7-diisopropyl pteridine phosphate) crystals were weighed and dissolved in acetone. Sterile Whatman filter paper discs containing 10 µg each of the vibriostatic compound were prepared. Discs were placed on agar plates seeded with the test organism and incubated. After incubation period, area of inhibition of growth around the discs was recorded as sensitive (Shewan *et al.*, 1954).

RESULTS

Based on the biochemical tests conducted it was seen that the major genera of bacteria isolated from marine ornamental fishes showing pathogenic conditions belonged to *Flavobacterium*, *Vibrio* spp., *Alcaligenes*, *Pseudomonas*, *Acinetobacter*, *Aeromonas*, *Serratia*, and to Enteroacteriaceae family.

The species of bacterial isolates identified and their sources are given in Table 4 and the percentage wise occurrence of bacterial genera is given in Fig. 13.

Biochemical characteristics of the isolates

The results of standard biochemical tests for all the isolates are given in Table 5. The important characteristics of the isolated genera are as given below.

Flavobacterium

A total of 27% of the isolates belonged to this genus. Out of the 12 isolates of *Flavobacterium*, 41.66% were from clown fish (Table 4, Fig. 13). These were yellow pigmented and penicillin sensitive. Other character, which helped in identifying the group, was the negative reaction to cytochrome oxidase test.

Vibrio

Three species, *Vibrio mediterranei, V. furnissi and V. fluvilais were* isolated from the ornamental fish in the present study. The three species together formed 18% of the isolates (Table 4, Fig. 13). 50% of the *Vibrio* isolates were obtained from clown fish. The *Vibrio* group was cytochrome oxidase positive and in general, showed fermentation in H&L medium without the production of gas, while *V. fluvialis*, exhibited alkaline reaction. H_2S

Table 4. Isolates of bacteria identified and source

Code	Identified genus/species	Host fish	Infection / Lesions in fish
9-98B	*Alcaligenes*	*Acanthurus*	Ulceration
10-98A	*Acinetobacter*	Serranid	Gill
15-98A	*Flavobacterium*	*Apogon*	Fin rot
15-98B	*Flavobacterium*	*Apogon*	Gill
20-98V	*V. furnissi*	*Acanthurus*	Ulceration
20-98D	*Alcaligenes*	*Acanthurus*	Ulceration
20.98W	*A. hydrophila*	*Acanthurus*	Ulceration
20-98R	*S. marcescens*	*Acanthurus*	Ulceration
23-98	*Alcaligenes*	Serranid	Ulceration
29-98	*Pseudomonas*	*Acanthurus*	Haemorrhages
40-98A	*V. fluvialis*	*Amphiprion*	Ulceration
40-98B	*Pseudomonas*	*Amphiprion*	Ulceration
40-98C	*A. hydrophila*	*Amphiprion*	Ulceration
40-98D	*Pseudomonas*	*Amphiprion*	Ulceration
42-98	*Alcaligenes*	*Acanthurus*	Ulcerations, haemorrhages
71-98A	*Flavobacterium*	*Amphiprion*	Unilateral exophthalmia
71-98B	*Pseudomonas*	*Amphiprion*	Unilateral exophthalmia
78-98	*V. furnissi*	*Amphiprion*	Fin rot
72-98	*Flavobacterium*	Damsel	Fin rot
103-98	*Alcaligenes*	*Amphiprion*	Haemorrhagic areas
103-98A	Enterobacteriaceae	*Amphiprion*	Haemorrhagic areas
103-98B	*Acinetobacter*	*Amphiprion*	Haemorrhagic areas
115-98	*Pseudomonas*	Damsel	Deep ulcers
1-99	*Acinetobacter*	*Acanthurus*	Fin rot, ulcerations
4-99	*Acinetobacter*	*Apogon*	Fin rot
41-99	*Pseudomonas*	Serranid	Ulcerations and fin rot
50-99A	*Flavobacterium*	Banner fish	Haemorrhagic areas, fin rot
50-99B	*V. mediterranei*	Banner fish	Haemorrhagic areas, fin rot
61-99	*Pseudomonas*	*Amphiprion*	Fin erosion, ulceration
61-99A	*V. mediterranei*	*Amphiprion*	Fin erosion, ulceration
61-99B	*V. mediterranei*	*Amphiprion*	Fin erosion, ulceration
68-99A	Enterobacteriaceae	*Chaetodon*	Ulcerations
68-99B	*Flavobacterium*	*Chaetodon*	Ulcerations
73-99	*V. mediterranei*	*Chaetodon*	Ulcerations
117-99	*Flavobacterium*	Squirrel fish	Ulcerations
124-99	*V. furnissi*	*Acanthurus*	Deep ulcerations
151-99A	*Acinetobacter*	*Chaetodon*	Fin rot
151-99B	*S. marcescens*	*Chaetodon*	Fin rot, severe ulcerations
151-99C	*Alcaligenes*	*Chaetodon*	Fin rot
CFY	Flavobacterium	*Amphiprion*	Ulcerations, fin rot
CY	*Flavobacterium*	*Amphiprion*	Ulcerations, fin rot
LJ	*Flavobacterium*	*Apogon*	Ulcerations
OE	*Alcaligenes*	*Apogon*	Ulcerations
8-2K	*Flavobacterium*	*Amphiprion*	Fin rot
9-2K	*Flavobacterium*	*Amphiprion*	Fin rot

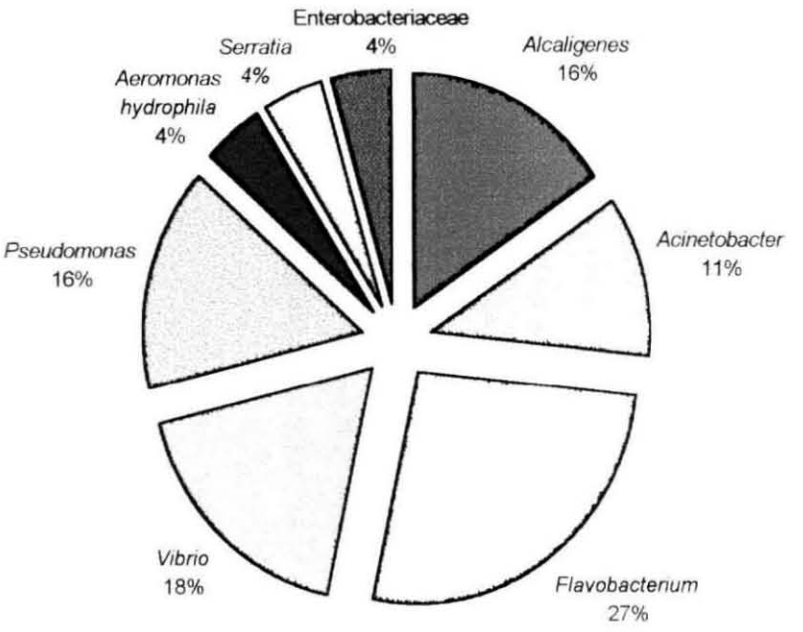


Fig. 13. Percentage composition of genera of bacterial isolates identified

Table. 5. Biochemical characteristics of bacterial isolates

Biochemical tests	*Alcaligenes*	*Acinetobacter*	*Flavobacterium*	*V. furnissi*	*V. fluvialis*	*V. mediterranei*	*Pseudomonas*	*A. hydrophila*	*S. marcescens*	Enterobacteriaceae
Penicillin	+	-	+	-	-	-	-	-	-	-
H&L	N	N	d	F	Alkaline	F	N	F	F	F
Oxidase	+	-	-	+	+	+	+	+	-	-
H_2S production	d	-	d	+	-	+	-	+	+	d
Indole	-	-	-	d	-	+	-	d	D	-
Starch	d	d	-	-	-	d	D	d	D	-
Gelatin	d	d	d	d	-	-	D	d	+	+
Glucose	d	d	d	A	-	d	D	A, G	A, G	A
Sucrose	d	A	d	A	-	A	D	A, G	A, G	A
Lactose	d	d	-	d	-	-	-	d	-	d
Sorbitol	d	-	-	d	-	D	D	-	A, G	-
Arabinose	D	-	-	d	-	-	D	+	D	A
Urease	D	d	d	-	+	+	D	d	D	d
Growth at 5°c	-	d	-	d	-	D	D	-	D	d
Growth at 37°C	+	+	+	+		+	+	+	+	+
Growth in 0% NaCl	D	+	+	+	+	D	D	+	+	+
Growth in 5% NaCl	+	+	d	+	+	+	D	-	D	+
Growth in 7% NaCl	-	S+	d	d	+	+	D	-	D	d
Citrate	+	+	d	+	+	-	D	d	-	d
Argininine	D	+	d	+	+	D	D	d	-	d
Lysine	D	+	d	d	-	-	D	d	+	d
Ornithine	D	+	d	+	-	-	D	-	+	d
Methyl Red	D	-	-	d	-	D	D	+	-	+
Voges-Proskaur	D	+	d	+	-	-	D	-	D	-
0/129	D	+	d	+	-	+	D	-	D	d
ONPG	+	+	d	+	-	D	D	d	+	+
Growth in TCBS	-	-	-	+	+	+	+	+	-	-
Motility	+	d	-	+	+	+	+	+	+	d

+ - More than 90% of identified genera show positive result; - - More than 90% of identified genera show negative result; d - 11-89% sh positive result; N - No reaction; A- Acid production; G- Gas production; F- Fermentation

production was exhibited by *V. furnissi* and *V. mediterranei* while for *V. fluvialis* it was absent. *V. furnissi* was urease negative, while the other two species of *Vibrio* were positive for urease. Growth at higher concentrations of sodium chloride (7%) were exhibited by *V. mediterranei* as well as *V. fluvialis*. Citrate utilisation was positive for *V. fluvialis* and *V. furnissi*. Except *V. fluvialis* the other two species showed sensitivity to 0/129 .

Alcaligenes

Alcaligenes was another dominant genus in the present study contributing to 16% of the isolates. Most of the isolates (43%) were from *Acanthurus* (surgeon fish) (Table 4, Fig. 13). The genus *Alcaligenes* showed motility and was cytochrome oxidase positive which were the characteristic features.

Pseudomonas

Maximum numbers of pseudomonads were isolated from clownfish (43%) (Table 4, Fig. 13). The characteristics helpful in identifying this genus were the penicillin resistance, negative response to cytochrome oxidase test and the absence of fermentation in H&L medium.

Acinetobacter

This genus formed about 11% of the total number of isolates and was associated with various groups of ornamental fish (Fig. 13). All the isolates were penicillin resistant, cytochrome oxidase negative as well as non-fermentative in H&L medium.

Aeromonas hydrophila

The occurrence of *A. hydrophila* was noticed in the marine aquarium in two cases forming 2% of the total number of bacterial isolates (Fig. 13). The species was identified based on the penicillin resistance, characteristic fermentation reaction with production of gas in H&L medium

Serratia marcescens

The *Serratia* genus was isolated for the first time from the marine aquarium. The species *S. marcescens* was isolated twice and was found to be associated with severe ulcerations of fish indicating that it was capable of high proteolytic activity, which was proved, later on by biochemical characterisation (Table 5) as well as *in vitro* studies. One important characteristic of the bacterium is the production of the red pigment (prodigiosin). Other features included the H_2S production, negative M-R reaction as well as Arginine negative, lysine and ornithine positive reactions.

Enteroacteriaceae

The isolates which were found to be penicillin negative, fermentative and Kovac's indole negative were classified as belonging to Enterobacteriaceae group and were represented by 2% of the isolates (Fig. 13).

DISCUSSION

Characterisation of marine bacteria has been a tough task and the ambiguity in identifying the strains was noticed for a long time. Identification of fish microflora has typically relied on phenotypical and biochemical key characteristics (Cahill, 1990 a), which is time consuming. According to Spanggaard *et al.* (2000), it is tempting to use limited number of key characteristics. In the present study, about 26 key characters were carried out to arrive at the genus and species level identification of important isolates (Table 5). Nevertheless, molecular biology tools were not used for identification. It was mentioned by Spanggaard *et al.* (2000) that high agreement was found between traditional and molecular identification of intestinal microflora of rainbow trout intestine. Hence, it can be presumed that the results of classical biochemical characterisation is sufficient to identify bacteria.

Predominant component of aerobic heterotrophic bacterial flora of seawater comprise, Gram negative asporogenous rods, motile usually by flagella (Pfister and Burkholder, 1965; Austin *et al.*, 1979a). In the present study, the isolates were obtained from lesions including fin erosions and various organs including liver, intestine, kidney etc. They included *Alcaligenes*, *Acinetobacter*, *Flavobacterium*, *V. furnissi*, *V. fluvialis*, *V. mediterranei*, *Pseudomonas*, *A. hydrophila*, *S. marcescens* and members of Enterobacteriaceae family (Fig. 13).

In general, the bacterial flora in fish reflects the flora of the environment (Horsely, 1973; Sakata *et al.*, 1980) and equate well with the populations in the surrounding water (Austin and Austin, 1987). The studies on indigenous microflora include the descriptions of microbial spoilage (Joseph *et al.*, 1988) as well as the relationship between environment and fish microflora (Horsely, 1973). As indicated by Munro *et al.* (1994) high rates of mortality are not necessarily associated with the presence of recognised bacterial pathogens.

As already observed, by various authors, the normal microflora become pathogenic under conditions of stress and become involved in disease processes and hence are frequently isolated from diseased or moribund fish. The external surface and the intestinal tract are possible locations of bacteria in normal cases and under pathogenesis, they are quite likely to be isolated from internal organs including liver, kidney etc.

The intestinal flora of fish received much attention by several authors (Sugita *et al.*, 1987; Westerdahl *et al.*, 1991; Ringø *et al.*, 1995). While species like *Acinetobacter, Alcaligenes, Pseudomonas and Vibrio* species were frequently isolated from surface of marine fish as reported by Austin and Austin (1987), species belonging to *Aeromonas* (Ugajin, 1979), *Vibrio* (Muroga *et al.*, 1987; Sakata *et al.*, 1980; Yoshimizu *et al.*, 1976) and *Pseudomonas* (Liston, 1957) genera have been regularly recovered from the intestine of older marine fish.

From a study by Tanasomwang and Muroga (1990), bacterial genera recovered from the intestine of larval and juvenile stages of red sea bream (*Pagrus major*), black sea bream (*Acanthopagrus schlegeli*), Japanese flounder (*Paralichthys olivaceus*), rockfish (*Sebastes schlegeli*), tiger puffer (*Takifugu rubripes*) and red grouper (*Epinephelus akaara*) included *Vibrio*, *Pseudomonas*, *Acinetobacter*, *Moraxella*, *Cytophaga* and *Alcaligenes*. In a study on the intestinal microflora of rainbow trout intestine, Spanggaard *et al.* (2000) observed that the dominant Gram negative microflora comprised *Citrobacter*, *Aeromonas* and *Pseudomonas*.

Bacteria identified from moribund fry and juveniles of marine fish hatcheries in Hawaii included *Pasteurella* sp., *Vibrio* spp., *Aeromonas* spp., *Chromobacterium* sp., *Flavobacterium* sp., *Pseudomonas* sp. and an uncharacterised myxobacteria-like agent (Brock *et al.*, 1993).

As shown in Fig. 13, the majority of bacterial isolates belonged to the *Flavobacterium* group. According to Nogi *et al.* (1997), the taxonomic status of these genera is still unsatisfactory. In their studies, orange or yellow-pigmented bacteria were isolated from the Japan and were characterised phenotypically. These strains were Gram negative, non motile and their DNA G-C contents ranged from 30 to 40 mol% which suggested that they were considered probable members of the genus *Flavobacterium*. Though isolated from salmonid fish infected with bacterial gill disease, their role as the causative agent of this disease has not been well demonstrated (Sanders and Fryer, 1988).

Isolations from five different environments of the Chile coast proved that along with *Pseudomonas* and *Vibrio*, *Flavobacterium* dominated the majority of the isolates (Prado *et al.*, 1992). They are reported widely from marine fish (Mudarris *et al.*, 1994; Kinnunen *et al.*, 1997), oysters (Ortigosa *et al.*, 1995), sea cucumbers (Ward-Rainey *et al.*, 1996) and penaeid prawns (Hameed and Rao, 1993). The *Flavobacterium* species are also reported to be aerobes and oxidase positive (Sanders and Fryer, 1988; Roberts, 1989) while according to Surendran (1980) they are oxidase negative. Roberts (1989) placed them under Gram negative aerobic rods of uncertain affiliation. In the present study, they were characterised by the oxidase negative reaction and penicillin sensitivity, along with the pigmentation.

Alcaligenes was another genus, which was frequently isolated from the diseased ornamental fish in the present study. Along with *Pseudomonas* and *Bacillus*, *Alcaligenes* comprised 77.8% of the denitrifying bacteria isolated from coastal and oceanic bottom sediments off the Japanese coast (Sugahara *et al.*, 1988). In the present study, *Alcaligenes* formed 16% of the total number of isolates and were identified based on the positive oxidase reaction and motility.

Acinetobacter genus formed 11% of the total number of isolates, which were characterised by the negative cytochrome oxidase reaction and non-fermentative nature in H&L medium (Surendran, 1980), which were the distinctive characters for identifying this genus in the present study. As evident from the studies of the past, this species was regularly associated with the fish flora.

Another study on the bacteria isolated from the coastal marine fish rearing unit showed that, five of the major phena beloged to *Acinetobacter calcoaceticus*, *Photobacterium phosphoreum* and *Vibrio* spp., phena intermediate between *Cytophaga*, *Flexibacter* and *Flavobacterium* and Gram variable rods (Austin, 1982). In the same study, taxa identified as pure cultures from within the lesions of moribund animals, included *Alteromonas* and unidentified Gram negative budding bacteria. These studies indicate that the representatives of normal microflora in healthy fish are isolated from diseased ones, which proves that most of these species are opportunistic pathogens. In the present study carried out in the marine aquarium, the identities of the isolates were comparable with the findings of the earlier studies.

In a survey in three fish farms with intensive culture of gilt-head sea bream (*Sparus aurata* L) in southwest Spain, the main pathogenic microorganisms isolated were *Vibrio* (67.8%), *Pseudomonas* (13.5%), *Photobacterium damsela* subsp. *piscicida* (6.7%), *Cytophaga/Flexibacter*-like bacteria (4.8%), *Aeromonas* (0.5%), and Gram positive bacteria (6.7%) (Balebona *et al.*, 1998), whereas another study on the microflora associated with healthy and diseased turbot (*Scophthalmus maximus*) from three farms in northwest Spain indicated that, in all three farms, a wide range of bacteria was found in healthy turbot with *Vibrio* (*V. splendidus*, *V. pelagicus*, *V. fischeri*, V. har*veyi* and *Vibrio* spp.) and *Pseudomonas* spp. being the predominant group comprising at least 80% of total bacterial isolates in each farm (Toranzo *et al.*, 1993). It was also seen that the same species were the most prevalent bacterial isolates that could be recovered from diseased turbot.

V. furnissi was another species, which contribute to a major part of isolate from diseased fish in the present study. Role of V. *furnissi* as a possible human enteric pathogen is being discussed (Dalsgaard *et al.*, 1997; Magalhaes *et al.*, 1993). Other pathogens isolated from fish are also reported as potential human pathogens by various authors (Vandepitte, *et al.*, 1983; Mualu and Ijumba, 1982). Recent studies point to the role of *A. hydrophila* also as a causative agent for foodborne gastreoenteritis (Thampuran and Surendran, 1998).

Growth at 4^{0}C, and with 6-8% (w/v) sodium chloride in a broth medium were necessary to differentiate *V. furnissi* strains which were isolated from European eel culture farm from *Aeromonas* species when the API 20E system was used (Esteve *et al.*, 1995). In the present study the key of Alsina and Blanch (1994) was employed for characterising the *Vibrio* group. From the findings of the present study also, the *V. furnissi* isolates were capable of growing in medium containing 5% sodium chloride and majority of the isolate grew in medium containing 7%sodium chloride. *V. mediterranei* has been reported by Lowrie and Borneman (2000) to be associated with corals. *V. mediterranei* is reported to exhibit positive results to indole and ONPG reaction and negative response to gelatin and citrate (Cerdà cuéllar *et al.*, 1997).

Vibrios are cytochrome oxidase positive, fermentative by the oxidative/ fermentative (O/F) glucose test and sensitive to vibriostatic compound and novobiocin (Sanders and Fryer, 1988) whereas *Aeromonas* is resistant to it. In the present study, however, *V. fluvialis*, which was another component of the microflora, was found to be resistant to both (Table 5). The *Pseudomonas* species is also distinguished by the strict aerobic nature as well as the oxidative metabolism in O/F medium as stated by Sanders and Fryer (1988) (Table 5).

In the present study, two isolates were identified as *Aeromonas hydrophila, which* were isolated from *Acanthurus* and *Amphiprion*. Incidence of motile aeromonads in raw marine fish has been reported by Thampuran and

Surendran (1998). *A. hydrophila* and other motile aeromonads were found to frequently involve in spoilage of marine fish (Len, 1987; Thampuran and Surendran, 1998). Recently, Candan *et al.* (1995) and Doukas *et al.* (1998) have also reported the occurrence of *A. hydrophila* in marine fish.

In the present study, 16% of the isolates belonged to *Pseudomonas* genus. *Pseudomonas* species are widespread in fish intestine (Austin and Al-Zaharani, 1988; Trust and Sparrow, 1974). They were identified based on the oxidase positive reaction and oxidative type of metabolism in H&L medium. As recorded by Hansen and Olafsen (1989), members of the genera *Pseudomonas*, *Alteromonas*, *Aeromonas*, and *Flavobacterium* were dominant on the surface of both cod and halibut eggs. *Pseudomonas* spp. are also reported to be the specific spoilage bacteria of iced fresh fish (Gram and Huss, 1996). According to Surendran and Gopakumar (1981) the native flora of oil sardine and mackerel consisted of *Pseudomonas* spp. *Moraxella* spp. *Acinetobacter* spp. and *Vibrio* spp.

The species of Enterobacteriaceae are well known inhabitants the intestine and are cytochrome oxidase negative and fermentative in O/F glucose medium (Sanders and Fryer, 1988). The members of the family Enterobacteriaceae, which are currently recognised as substantiated fish pathogens, are *Yersinia ruckeri*, *Edwardsiella tarda* and *E. ictaluri*. Other members of the family including *Proteus*, *Citrobacter*, and *Hafnia* and *Serratia* species are occasionally reported to be associated with fish diseases (Llewellyn, 1980; McIntosh and Austin, 1990; Nieto *et al.*, 1990, Gelev *et al.*, 1990; Rodriguez *et al.*, 1998).

In the present study, the occurrence of *S. marcescens* in the infections of marine ornamental fish was significant and is reported for the first time from marine ornamental fish. In diseases processes of *Amphiprion*, *Chaetodon* and *Acanthurus*, which form a major portion of the ornamental tropical marine group, this genus was involved (designated as 20-98R and 151-

99B), leading to ulcerations and fin erosions. *Serratia* genus was isolated from salmonids (Llewellyn, 1980; Nieto *et al.*, 1990) as well as non salmonids (Baya *et al.*, 1992; Vigneulle and Baudin Laurencin, 1995. According to Austin and Gibb (1993), recent trends have highlighted the emergence of many Gram negative bacterial pathogens including *S. liquefaciens*, *S. marcescens* and *S. plymuthica* among the enteric pathogens. These species are reported as potential pathogens by many recent authors (Baya *et al.*, 1992; Vigneulle and Baudin Laurencin, 1995; McIntosh and Austin, 1990). Studies of Baya *et al.* (1992) to compare the virulence properties of a white perch isolate of *S. marcescens* with the reference strains proved that it could be highly pathogenic for fish. Vigneulle and Baudin Laurencin (1995) isolated *S. liquefaciens* from the farmed turbot *Scophthalmus maximus* and suggested that this species could be an opportunistic pathogen. McIntosh and Austin (1990) reported that an extremely proteolytic form of *S. liquefaciens*, is pathogenic to Atlantic salmon and have stated that this species could be a potential and serious pathogen for these fishes. According to Austin and Austin (1989), also the species is an emerging pathogen of its own weight.

It was suggested by Ewing *et al.* (1959) that it was on the basis of pigment formation and rapid liquefaction of gelatin that the group serratiae was recognised. According to the same authors, only one species was sufficient for taxonomic studies of this group, and cultures labeled with various names could not be distinguished from the key species, *S. marcescens*. Sakazaki (1974) also could not distinguish these species and considered *S. essyana*, *S. fuchsina* and *S. pyoseptica* to be *S. marcescens*.

The results of biochemical tests (Table 5) indicated that the *S. marcescens* isolates were penicillin positive, cytochrome oxidase negative, lactose negative as well as MR-VP negative. The cytochrome oxides negativity excluded the present isolate from common fish pathogenic organisms such as *Vibrio*, *Pseudomonas* and *Aeromonas* and *Alcaligenes*.

However, similar results were also obtained for other bacterial isolates such as *Serratia*, Enterobacteriaceae, *Acinetobacter* and *Flavobacterium*. The isolate 20-98 R, was negative for methyl red test whereas, generally for Enterobacteriaceae it is positive. *Serratia* was found to be resistant to penicillin whereas *Flavobacterium* was sensitive. In almost all the repeated isolations of *Serratia* strains, the liquefaction of gelatin was one of the important characteristics, whereas in a closely resembling species *Acinetobacter*, only a few isolates gave positive results. Similarly in the H&L test, the present isolate was fermentative while it was non fermentative in *Acinetobacter*. These characteristics suggest that the isolate can be grouped under *Serratia* spp.

The physical and biochemical features of the isolate are given in Table 5. As per Bergy's manual of determinative bacteriology (Sakazaki, 1974) the *Serratia* strains are motile and flagellated rods. The methyl red test is negative while the Voges-Proskaur test is usually positive. *Serratia* produces pigments. In the present isolates, identified as *Serratia*, prolonged incubation resulted in increased intensity of the pigment. In the Bergy's manual only one species, *S. marcescens* was described while subsequently, fish pathologists isolated and identified the virulence properties of other species including, *S. liquefaciens* and *S. plymuthica* from fishes (Vigneulle and Baudin Laurencin, 1995; McIntosh and Austin, 1990; Nieto *et al.*, 1990). Between the two species *S. marcescens* and *S. plymuthica*, *S. marcescens* was considered as a potential pathogen for fish by Baya *et al.* (1992) who isolated red-pigmented bacterium, which resembled *Serratia* species in pure culture from 20% of the white perch, *Morone americanus*, sampled from Back River, Maryland, USA. The isolate had the following characteristics, Gram negative motile rods, fermentative, oxidase and catalase negative, indole negative, Voges-Proskauer and nitrate positive, arginine dihydrolase negative but lysine and ornithine positive and gas production from glucose, but not H_2S positive, all of which were found to be similar to that of the ATCC reference strain. The proteolytic activity such as caesinase and gelatinase were also reported, in which it deviated from the reference strain. However, in the present isolate of *Serratia*, H_2S production was

observed. All the other characters resembled the white perch isolate of *S. marcescens* reported by Baya *et al.* (1992).

From the results of the present investigations and from the available information, it could be inferred that most of the bacterial isolates from the ornamental marine fishes are comparable with those, which were reported earlier. Isolation of the potential pathogenic species, *S. marcescens*, which was involved in the pathology of *Acanthurus* and *Chaetodon*, can be considered as the emergence of a possible pathogen associated with the marine aquarium fishes. In view of the pathological activities displayed by these isolates, more studies on precise taxonomic status are essential. The other major bacterial genera isolated from marine ornamental fish in the study was in accordance with the observation of various authors as explained above, as well as with results obtained by Austin and Austin (1989) according to whom the fish body surface maintains microflora containing *Acinetobacter*, *Flavobacterium*/ *Cytophaga*, *Moraxella* and *Pseudomonas*, and the intestinal tract harbours representatives of family Vibrionaceae. Considering their isolations from diseased fish, it can also be inferred that these isolates are opportunistic pathogens under favourable conditions.

CHAPTER 3

STUDIES ON THE PATHOGENICITY OF *SERRATIA MARCESCENS* TO CLOWNFISH, *AMPHIPRION SEBAE*

The pathogenicity and the true pathogenic nature of the bacterial isolate has to be determined by Koch's postulates. According to Koch's postulates, the disease-causing organisms are involved uniformly in all cases of a disease outbreak, and can be isolated in pure form from the diseased organism, which when administered to a normal healthy host, is capable of inducing similar type of lesions which can be again isolated from the experimentally infected host.

The bacterial pathogen *Serratia marcescens*, which was associated with pathological conditions of commercially important marine ornamental fishes, was selected for investigating the role in inducing pathogenicity in experimental host. Based on the preliminary *in vitro* and *in vivo* observations, it was proved that this pathogen had highly proteolytic Extracellular products (ECPs) as well as inflicts significant lethality to Tilapia, *Oreochromis mossambicus*. However, *S. marcescens* was not previously reported from marine aquaria. Considering this lacuna as well as to substantiate the role of the isolate in causing diseases, the pathogenicity was determined.

The circumstances under which the host succumb, vary depending on the virulence of the pathogen, number of cells required to bring about lethality or the nature of toxic principles along with other physico-chemical factors prevailing in the system. ECPs, which are produced during the course of infection and pathogenesis are involved in causing pathogenicity. Hence, the infectivity of live cells as well as the role of cell free extract (ECP), in causing lethality in the experimental host, *A. sebae* were studied, by *in vitro* and *in vivo* methods. In addition, characterisation of the proteins responsible for pathological symptoms by sodium dodecyl sulphate polyacrylamide agar gel

electrophoresis (SDS-PAGE) was also carried out.

Pathogenicity due to Bacterial infections

As stated earlier, the disease causing bacteria of aquaculture belong to some important genera of certain families of aquatic microflora. According to Sindermann (1990), bacterial pathogens, which cause haemorrhagic septicaemia, are of greatest importance despite the difficulties in identification and taxonomy. Knowledge concerning the pathogenesis of many bacterial diseases in fish is limited, especially in those diseases that occur in warmwater species, which is due to the relative recent emergence of warmwater fish culture as a major industry in many parts of the world, and due to the previous economic insignificance of warmwater aquaculture and the bacterial pathogens affecting warmwater species (Thune *et al.*, 1993). Bacterial diseases of marine tropical fish have been less extensively studied (Stoskopf, 1993).

Although there are several bacterial diseases among the marine, estuarine and fresh water fishes (46 genera as listed by Adams *et al.*, 1999), the exact etiology of some of the recent and emerging diseases have not been determined. One of the important criteria to determine the pathogenicity is to conduct experiments and check for their virulence (Kevin, 1985).

Both extracellular products as well as whole cells of bacteria are responsible for pathogenicity in the case of bacterial pathogens leading to various types of host-pathogen interactions. Since many pathogens produce proteolytic enzymes during the course of infection and pathogenesis, it is believed that knowledge concerning the nature and role of antiprotease in fish would further help in understanding of host-pathogen interactions (Ellis and Grisley, 1985). Pollack (1962) defined extracellular (exocellular) enzymes as those enzymes, which are found free in the medium. Many workers carried out studies on the aspects of pathogenicity due to ECP of bacterial isolates, which are commercially significant in aquaculture practices.

Vibrio

A potential virulent protease factor was described by Norqvist *et al.*, (1990) for *V. anguillarum* and the secretion of an extracellular toxin, whose toxicity was associated with two 44 kDa and a single 34 kDa protein was reported by Kodama *et al.* (1985). Inamura *et al.* (1984, 1985) suggested that a protease of molecular weight 36 kDa was responsible for lethality of *V. anguillarum* in goldfish where the LD_{50} value was 1.7µg/g of fish and established that the exotoxins were heat labile. A representative strain from salmonids, *V. marinus* is demonstrated to be having LD_{50} value lower than 3.5×10^3 (Benediktsdóttir *et al.*, 1998). Swab treatments of *V. damsela* with 10^7 to 10^8 cells were found to lead to ulcerations in damselfish, *Chromis punctipinnis* and found pathogenic in four other species of damsels (Love *et al.*, 1981). Its pathogenicity is associated with the production of a cytolysin named damselysin (Kreger, 1984).

In another study, Fouz *et al.* (1993) conducted the *in vivo* and *in vitro* activities of ECPs of several isolates of *V. damsela* for both fish and established that the ECP from all the strains were strongly lethal for fish, with LD_{50} values ranging from 0.06 to 3.7 µg protein/g fish. These ECP samples possessed remarkable phospholipase and hemolytic activity and the levels of enzymic and cytotoxic activities were associated with the degree of virulence for fish. It was also observed that the *in vivo* and *in vitro* biological activities were considerably reduced after heat treatment (100^0C for 10 min), but not totally lost in the highly virulent strains.

Many authors demonstrated the occurrence and pathogenicity aspects of opportunistic pathogens. Saeed (1995) reported that Silvery black porgy, *Acanthopagrus cuvieri* was susceptible to laboratory infection with *V. harveyi* isolate, associated with mortalities of this fish, only when administered intramuscularly (IM). But the isolate could infect brown-spotted grouper by both IM and intraperitoneal (IP) routes. The high LD_{50} values (5 day LD_{50} for silvery black porgy was $4.9\pm0.21\times10^7$ CFU; and for brown-spotted

grouper, $1.56\pm0.19\times10^9$ CFU for IM and $1.59\pm0.17\times10^9$ CFU for IP infection) indicate the opportunistic nature of the pathogen. However, the involvement of an extracellular product extracted from a broth culture of the bacterium in the pathogenesis of the infection was ambiguous.

Aeromonas

Significant amount of work has been done on the pathogenicity aspects of *A. salmonicida*. In general, the disease causing factors are considered mainly the ECP and cell surface structures. (Hiney and Olivier, 1999). Effects of ECPs in infection in susceptible fish have been demonstrated by various authors (Ellis *et al.*, 1981, Ellis, 1991). A lethal protein of 70kDa was first detected in *A. salmonicida* by Tajima *et al.* (1983) and the same was found to have an LD_{50} value of 2.4 μg/g. But the role of this protein in pathogenicity was questioned by others (Hackett *et al.*, 1984, Ellis *et al.*, 1988) with identification of a virulent isolate, which did not have this protein. Later it was observed that *under in vivo conditions* the same isolate produced the protease (Ellis, 1991). Price *et al.* (1989) recorded the occurrence of proteins with 70 kDa, as a serine protease and were detected to be active against gelatin.

Atypical strains of *A. salmonicida* were found to be toxic to a wide variety of host species (Bucke, 1980). In salmonids, LD_{50} values ranging from 50 cells to 10^{2-3} cells were demonstrated (Olivier, *et al.*, 1990, Gudmundsdóttir *et al.*, 1997). Iida *et al.* (1997) recorded that intraperitoneal injection confirmed the virulence of atypical *A. salmonicida* to Japanese flounder.

In the case of *A. hydrophila* a variety of virulence factors including endotoxins, ECPs including cytotoxins, enterotoxins, hemolysin, proteases, hemagglutinins, acetyl cholin esterases etc have been suggested (Aoki, 1999; Cahill, 1990 b). Boulanger *et al.* (1977) isolated two different hemolysins responsible for the pathogenicity. Stevenson and Allan (1981) also investigated the production of protease and hemolysin in the ECPs and demonstrated that the ECPs are analogous to those produced by *A. salmonicida* and could

produce effects on injected fish. The heat labile nature of ECP was also detected in the same study. According to Wakabayashi *et al.* (1981), proteases were found to be playing an important role in the pathogenicity of *A. hydrophila*. A metalloprotease and serine protease with molecular weights of 38 kDa and 22 kDa were found to have a lethal dose of 150 ng/g for fish and were found to be stable at 56^{0}C for 10 minutes (Rodriguez *et al.*, 1992).

Santos *et al.* (1992) evaluated the *in vivo* and *in vitro* biological activities of the extracellular products (ECP) from strains of *V. anguillarum* and *A. hydrophila with* different degrees of virulence for fish, with ECP containing moderate levels of protein (0.07 to 0.80 mg/ml) and were found strongly toxic for rainbow trout. The median lethal dose for *A. hydrophila* ranged from 1.88 to 3.62 µg ECP per gram of fish and from 4.72 to 6.36 µg ECP per gram of fish for *V. anguillarum* strains. Inoculation of the ECP from non-virulent and low-virulent strains also caused fish mortality. They also found that although most of the ECP samples displayed amylolytic, proteolytic, haemolytic, cytotoxic and dermatotoxic activities, these activities were reduced after heating at 56^{0}C for 30 minutes and totally lost by heating at 80 ^{0}C for 10 minutes.

Renibacterium salmoninarum

Results of artificial infections studies with *R. salmoninarum*, the causative agent of bacterial kidney disease, are given by Bruno (1986) and Gutenberger *et al.* (1991), by intraperitoneal injection of live or formalin killed cells. Both histological and hematological studied were carried out and was found to be an invasive intracellular organism (Bruno, 1986).

Flavobacterium/Flexibacter

Differences in strain virulence of *Flexibacter columnaris* have been reported by Pacha and Ordal (1970). Production of protease as the cause of pathogenicity was reported for *F. columnare* and *F. psychrophilum* (Bertolini *et al.*, 1994). Heo *et al.*, (1990) correlated the pathogenicity of

F. branchiophilum with the ECPs produced by the bacteria. Handlinger *et al.*, (1997) reported that symptoms of experimentally induced diseases in salmonids and non-salmonids in Tasmanian aquaculture were similar to that of natural infections by *F. maritimus*.

Yersinia ruckeri

Information is scanty regarding the virulence factors of *Y. ruckeri*, which causes the enteric redmouth disease. This could be due to the different serogroups associated with this pathogen and presence of virulent and avirulent strains (Horne and Barnes, 1999). Bullock *et al.* (1978) also reported that serological variations between strains could be the reason of differences in pathogenicity.

Edwardsiella

Most pathogenicity studies of these bacteria were conducted in eels and channel catfish. (Plumb, 1999). Exotoxins, hemolysins and dermatotoxins were reported to be responsible for pathogenicity (Ullah and Arai, 1983). Heat lability of the ECPs of virulent strain of *E. tarda* was reported by Suprapto *et al.* (1995). Intraperitoneal injection with this pathogen has demonstrated the pathogenicity of this species for salmonids (Amandi *et al.*, 1982).

Newton *et al.*, (1989) demonstrated that characteristic symptoms were exhibited by 93% of the host fish on exposure to 5×10^8 cells, based on pathological studies conducted with *Edwardsiella ictaluri*. Susceptibility of five species of fingerling fishes to *E. ictaluri* was tested by Plumb and Sanchez (1983) with live cells, of which channel catfish was highly susceptible.

Enterococcus / Streptococcus

Studies on cocci pathogenic to fish were documented by Kimura and Kusuda (1982), in which toxic fractions of exotoxins, E and G of

Streptococcus play a significant role in pathogenesis. Among these, hemolysins were reported to be involved in infections as well as pathogenicity. Studies by Kusuda and Kimura (1978) with ECP confirmed the hemolytic activity and its role in inducing clinical signs in yellowtail, *Seriola quinqueradiata*. The lethal factors were found to be proteinaceous as the lethality was lost on heat treatment (Kusuda and Hamaguchi, 1988). Kawahara *et al.* (1989) suggested that the medium used and phase at which cells were harvested also determines the toxicity. Evans *et al.* (2000) conducted experimental infection of hybrid striped bass (*Morone chrysops*× *Morone saxatilis*) and tilapia (*Oreochromis niloticus*) with *Strepococcus iniae*, by nares inoculation with live cells and found that at least 4.8×10^3 colony-forming units were required to initiate infection for both species.

Mycobacterium

Inducement of infection by culture of *Mycobacterium* at 10 and 1 mg/ litre demonstrated 100% lethality after 14 and 30 days of injection, with signs of characteristic granulomatous lesions (Hatai *et al.*, 1988). Other studies by Arakawa and Fryer (1984), demonstrated the effect of temperature on mortality. The protein profiles of ECPs of *Mycobacterium* species by SDS-PAGE studies with *Mycobacterium* spp. isolated from freshwater ornamental fish indicated major bands at 65 and less than 14 kDa (Chen *et al.*, 1997).

Pseudomonas and related genera

The pseudomonads are associated with both healthy and diseased fish. An isolate of *Pseudomonas anguilliseptica* from European eel, *Anguilla anguilla* L. was tested for pathogenicity by experimental infection in post elvers and larger European eels, and showed low pathogenicity (Haenen and Davidse, 2001).

Pseudoalteromonas piscicida was associated with mortalities of eggs of two pomacentrids, *Amphiprion clarkii* and *Amblyglyphiodon curacao* (Nelson and Ghiorse, 1999).

Little is known about the virulence mechanisms of *Photobacterium damsela*. However, the ECPs of *Photobacterium damsela* subsp. *piscicida* produced phospholipase and hemolytic activity among the host fish (Magarinos *et al.*, 1992).

Enterobacteriaceae

As stated, many bacterial pathogens including members like *Proteus, Citrobacter, and Hafnia* and *Serratia* species were occasionally reported to be associated with fish diseases (Llewellyn, 1980; McIntosh and Austin, 1990; Nieto *et al.*, 1990, Gelev *et al.*, 1990; Rodriguez *et al.*, 1998), which are gaining importance in aquaculture.

Serratia species, including *S. liquefaciens, S. marcescens* and *S. plymuthica* have been associated with bacterial septicaemia and mortalities in salmonids. Variable results of mortality and clinical signs were reported by McIntosh and Austin (1990) on injection of *S. liquefaciens*. Another species, *S. plymuthica* gave a mean lethal dose of 10^5 cells in experimental infection among rainbow trout (Nieto *et al.*, 1990). *S. marcescens* was also demonstrated to be a fish pathogen (Baya, *et al.*, 1992), which gave LD_{50} values of 5×10^3 cells to 10^5 cells for rainbow trout and striped bass respectively, by either intraperitoneal or intramuscular injection. But, fish were found to die sooner when intramuscular injection method was adopted. The ECPs of this bacterium was lethal for fish with mean LD_{50} values ranging from 0.22 to 4.8 μg protein per gram of fish (Baya *et al.*, 1992). It was also noted that the ECP from strains with high proteolytic activity were cytotoxic for fish, but the activity was lost on heating at 100^0C for 10 minutes. Pathogenicity tests conducted by Vigneulle and Laurencin (1995) indicated that the bacterium could be an opportunistic pathogen. Inoculation studies leading to clinical signs within 2-7days were

conducted and rainbow trout was found to be resistant to infection (Llewellyn, 1980).

The strains of *Hafnia alvei* were reported to influence high to moderate mortality among brown trout, *Salmo trutta* and have LD_{50} values ranging from 1.3×10^{4} to 2.5×10^{7} bacteria per fish (Rodriguez, *et al.*, 1998).

In addition to these bacterial pathogens, both Gram negative and Gram positive bacteria and previously unidentified ones are being reported from different parts of the world, most of them being opportunistic pathogens associated with infections when fish are under stress.

Pathogenicity studies with live bacterial cells

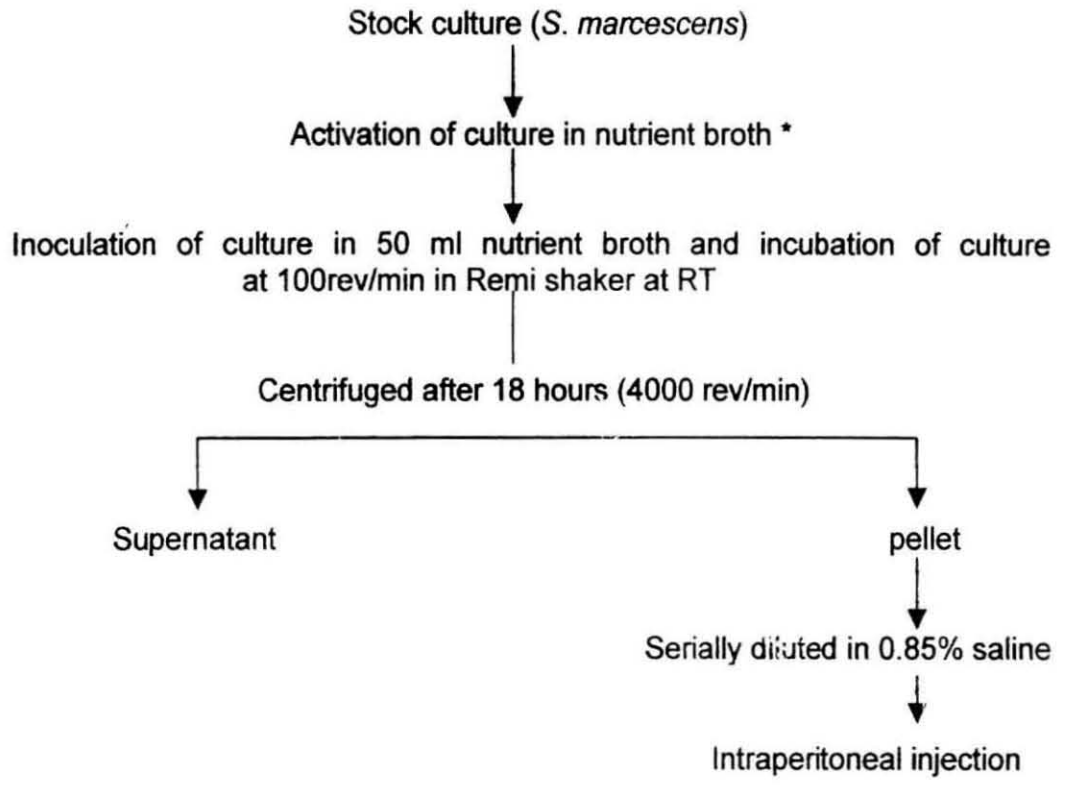
Bacterial isolate: The isolate, 20-98R identified as *S. marcescens* was used for studying the pathogenicity because of its repetitive occurrence in causing infections among Pomacentrids and also based on high protein hydrolysis activity recorded in the *in vitro* experiments.

Experimental set up: Specimens used for the experiments were *A. sebae* Bleeker collected from Gulf of Mannar coast and transported to the laboratory by oxygen packing. They were given an acclimatisation period of two days in 1 ton stocking tanks. One day prior to the experiments, they were transferred to the experimental glass tanks of 75 litre capacity. Both experimental tanks as well as stocking tanks were 'seasoned' prior to the introduction of the test fish. All the tanks were provided with bio filter and water recirculation was brought about by airlift through the filter (Plate 5).

Three different concentrations of the live cells were used for pathogenicity studies, viz. 10^5 cells, 10^6 cells and 10^7 cells. The cells were administered as intrperitoneal injection (IP) using an insulin syringe (29G×½) with low dead space integral needle (0.33×13mm) (Manufactured by Hindustan Syringes and Medical Devices Ltd., India). Control fish were given injection with 0.85% saline. Following injection of fish with the live cells, the behavioral response and the mortality pattern of the fish were closely monitored. The median lethal dose required for mortality was estimated graphically from probit chart (Miller and Trainter, 1944; Reed and Muench, 1938).

The isolates were tested for their pathogenicity in the experimental fish using median lethal time at the different cell densities mentioned above and the lethal dose was estimated from from probit analysis (Miller and Trainter, 1944). The flow chart depicting the method used is given below.

Plate 5. Experimental conditions for conducting pathogenicity studies



*Compositon(g/litre):Peptic digest of animal tissue – 5.0, Sodium chloride-5.0, Beef extract – 1.5, Yeast Extract –1.5; pH at 25^0C –7.4 ± 0.2.

Bacterial Growth Studies

The growth pattern of the selected isolate of bacteria (*S. marcescens*) was studied in detail. Growth of the bacterial cells was measured by three different methods (Lakshmanan *et al.*, 1971).

1. Direct count using haemocytometer
2. Total Plate Count method (viable count) and
3. Spectrophotometric method

The numbers of cells were calculated after measuring the sample intensity or cells count at intervals of 0, 3, 6, 9, 12, 15, 18, 24, 48, 72 and 96 h after inoculation of the cells in fresh medium, i.e., at each 3 hour interval of the incubation period, cells were harvested by centrifugation. The pellet was serially diluted and total count was taken in Neubaur counting chamber. For viable

count, 0.1 ml from the dilution was spread plated on agar plates and incubated. For spectrophotometric (tubidimetry) method, the absorbance was read at 530nm in a spectrophotometer against a dilution blank.

The count was plotted against time for estimating growth pattern. The generation time (GT) of the bacteria was calculated by plotting the growth curve. Generation time (GT) of the isolate was calculated using the following formula.

$$GT = T/N$$

T=Time interval in minutes for which GT is required

N= Number of generations

$$N = \frac{\log b - \log a}{\log 2}$$

Log b=log number of organisms at completion of time T

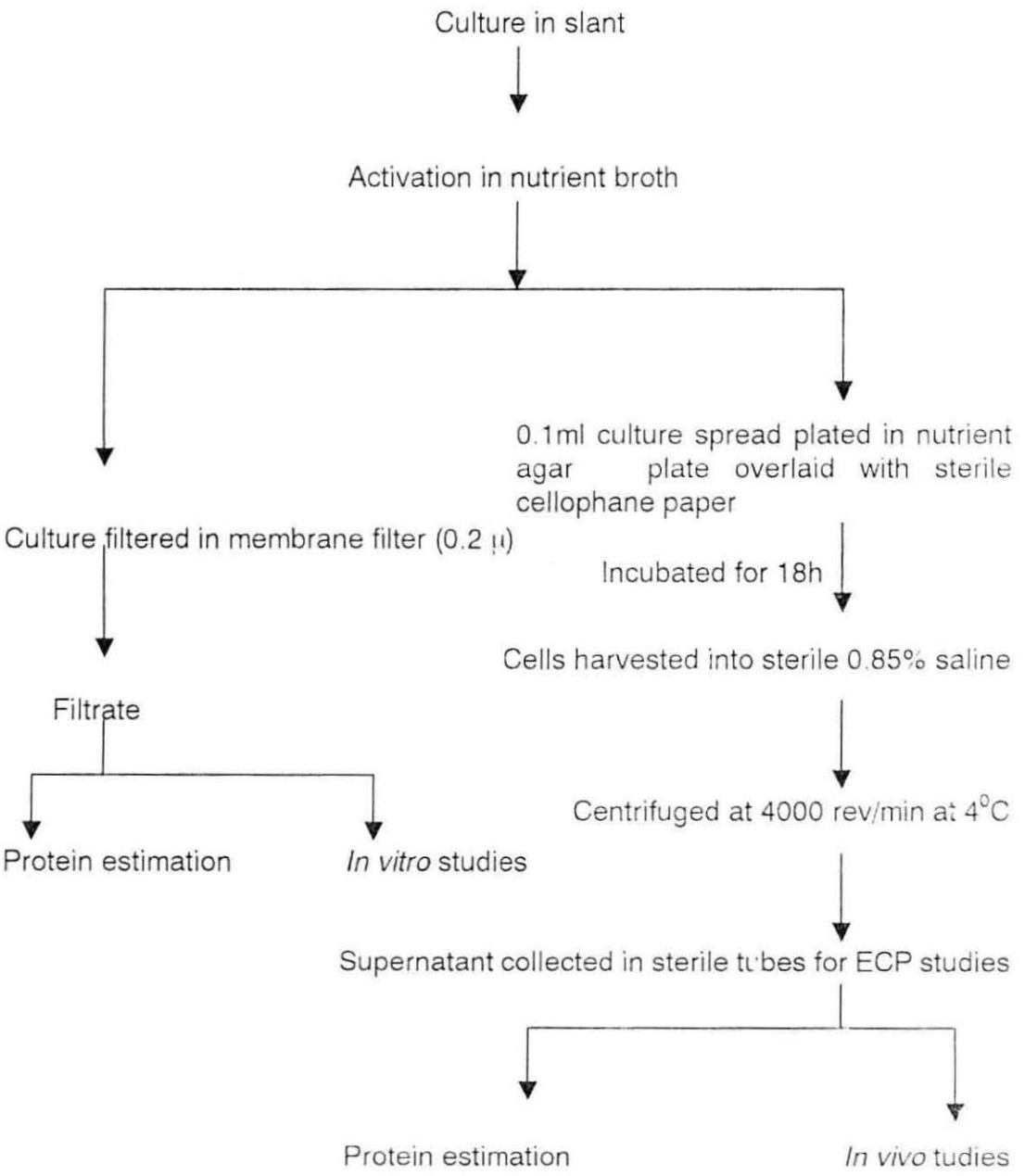
Log a= log number of organisms at the beginning of time T

Studies on Exocellular Products (ECP) of Bacterial Pathogen

Exocellular products of the selected bacterial isolate was obtained by the following two methods:

1. Cell free culture (Culture filtrate) method: The ECP was extracted from the broth culture at 0, 3, 6, 9, 12, 15, 18, 24, 48, 72 and 96 hours using membrane filter (0.2 μ, Millipore) by filtering the broth culture with a syringe adapter fitted with the filter.
2. Cellophane membrane method: The cellophane overlay method of Liu (1957) was followed for extracting the exocellular products free of media components.

The scheme for preparation of ECP via both the methods is given in the following flow chart.


Culture in slant
Activation in nutrient broth
0.1ml culture spread plated in nutrient agar plate overlaid with sterile cellophane paper
Culture filtered in membrane filter (0.2 μ)
Incubated for 18h
Cells harvested into sterile 0.85% saline
Filtrate
Centrifuged at 4000 rev/min at 4°C
Protein estimation
In vitro studies
Supernatant collected in sterile tubes for ECP studies
Protein estimation
In vivo tudies

Protein estimation of ECP

Protein estimation was carried out for the filtered as well as ECP extracted by cellophane technique by the Lowry's (1951) method.

In vitro studies with filtered ECP

The proteolytic activity of the bacterial ECP extracted by the filtration method against the substrates (casein and gelatin were used as substrates) was studied by incorporating these in agar media, by well diffusion method. After pouring the medium and allowing it to set, wells were cut in the medium and the bottom of each well was sealed with 1% agar.

ECP was inoculated in the well and incubated at 37^0C for 24 hours. After incubation period, saturated ammonium sulphate solution or acid mercuric chloride solution was poured into the plates. Zones of clearing around the wells indicated areas of hydrolysis of protein. The diameter of the zone in each substrate was measured and expressed.

Thermolability of ECP

In order to find out the thermostability or lability of ECP, experiments were conducted with control and heat-treated ECP. ECPs extracted at 6 h and 24 h were used for testing the activity, based on the results obtained in protein estimation of filtered ECP. The ECP was allowed to remain in water bath at 50^0C for 10 minutes. The ECP thus treated was used for the hydrolysis activity, following the above methodology.

Pathogenicity studies with ECP extracted by cellophane overlay technique

For estimating the median lethal dose value for the ECPs, extracellular products extracted by the cellophane overlay culture method (Liu, 1957 and Liu *et al.*, 1996) as described elsewhere in the thesis was used.

After estimating the protein concentration of ECP, doubling dilutions of the ECPs a the rate of 0.2ml/fish were administered using an insulin syringe (29G×½) intraperitoneally to experimental clown fish at three different protein concentrations (29.4, 14.7 and 9.8μg / fish). Control fish were given 0.1 ml saline injection. Experimental fish were closely observed for behavioral changes after injection, up to 24 h. The dose required for 50% mortality (LD_{50}) was found out from the probit chart (Miller and Trainter, 1944; Reed and Muench, 1938). The median lethal time taken for mortality (LT_{50} value) with the ECP administration was also estimated by plotting a graph with time taken for mortality in the x-axis and percentage mortality in the Y-axis.

Molecular Weight Determination Of ECP

As protease activity and toxicity studies in experimental fish were confirming the lethal influence of ECPs, the molecular weight of the protein involved has to be evaluated. The ECPs were extracted at different time intervals by the cellophane overlay culture method in ordinary nutrient agar and in nutrient agar with 3.5% sodium chloride in order to find out the expression of protein at different time intervals as well as to find out the effect of increased salt concentration in the culture medium, since the source of the isolate is a marine fish. SDS-PAGE (Laemmli, 1970) was conducted to determine the molecular weights of the exocellular proteins, with standard molecular weight markers of 14 KD to 100 KD (Bangalore Genei).

11.5% stacking gel and 6% separating gel were used. 10μl of SDS marker and 10 μl sample buffer with 2%SDS were boiled for one minute and loaded in to the wells. The proteins were stained with Coomassie brilliant blue stain and destained with methanol (10%) acetic acid (7%)mixture.

The Rf value (relative mobility) of the protein was obtained by the following formula

$$\text{Rf value} = \frac{\text{Solute front}}{\text{Dye front}}$$

The resultant figure and the molecular weights of the standard markers were plotted on a semi log graph with Rf values in the X axis and molecular weights in the Y-axis. From the graph, molecular weights of the ECPs were estimated after calculating their Rf values.

RESULTS

Pathogenicity Experiment with Live Bacterial Cells

The lethal dose (LD_{50}) value of the live cells for the experimental fish was found out from the probit chart. The percentage mortality for different cell densities is given in Table 6.

From the probit chart (Miller and Trainter, 1944) the LD_{50} was found to be 5.01×10^5 cells (Fig.14.), i.e. per gram of fish 1×10^5 cells were required to bring about 50% mortality. The median lethal time (LT_{50}) taken for 50 % mortality with administration of cells at the rate of 10^7 cells was estimated to be 45 h from the graph.

Other behavioral and morphological changes in fish after intraperitoneal injection were also noticed. The stress brought about by injection of the fish led to certain behavioral changes in the fish. They were found hiding in the anemone with reduced activity and took no feed (anorexic condition).

In addition, the area around the site of injection showed slight reddish coloration, which was also observed in the opercular region irrespective of the dose injected (Plate 6). Some of the injected fish showed caudal and other fin erosion. Another visible change in the injected fishes were the exophthalmic conditions exhibited by majority of them (Plate 7).

Bacterial Growth Studies

The total bacterial cells obtained by direct count by haemocytometer, total plate count and optical density values read in spectrophotometer are expressed in Table 7.

Table 6. Percentage mortality of *A. sebae* challenged with the pathogenic bacterial isolate, *S. marcescens*

Cell count (Cells/ml)	Percentage mortality in	
	Experiment	Control
10^5	20	0
10^6	60	0
10^7	100	0

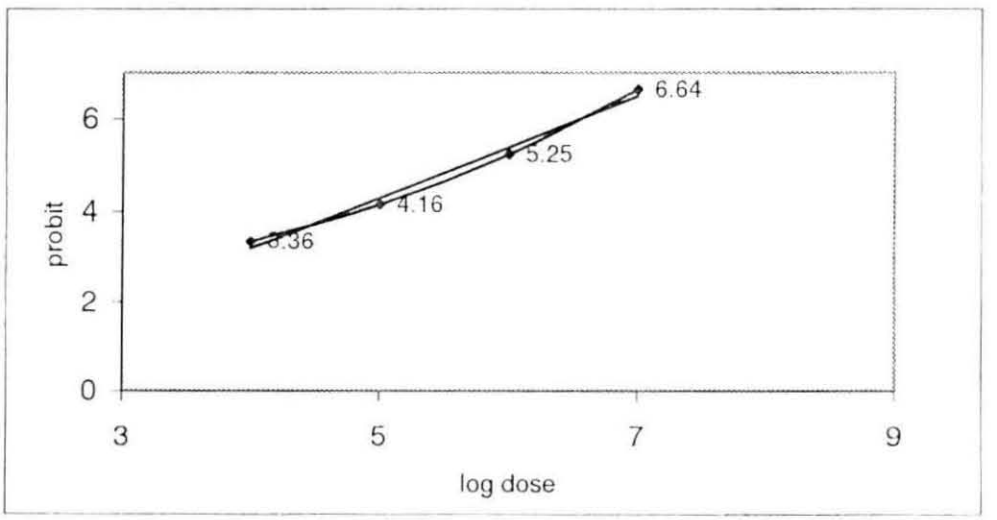


Fig. 14. Probit chart for estimation of median lethal dose of the live cells of *S. marcescens*

Plate 6. Experimentally infected *A. sebae* with reddish colouration on opercular region

Plate 7. Experimentally infected *A. sebae* with exophthalmia

Table 7. Growth of *S. marcescens* isolate at different time intervals

Time interval	Direct count	TPC	O.D. value (10^{-1} dilution)
0	6.5×10^{8}	3.45×10^{6}	0.039
3	3.61×10^{9}	4.8×10^{7}	0.03
6	4.755×10^{9}	1.2×10^{8}	0.4
9	4.8×10^{9}	2.61×10^{8}	0.41
12	5.12×10^{9}	3.09×10^{8}	0.48
18	6.63×10^{9}	2.16×10^{9}	0.65
24	4.435×10^{9}	2.04×10^{9}	0.089
48	3.085×10^{9}	1.92×10^{9}	*
72	2.885×10^{9}	1.8×10^{9}	*

* not read

The growth patterns of the bacteria by the direct count and total plate count are shown in Fig. 15 and 16.

The growth of the isolate was found to follow the sigmoid pattern as indicted in Fig 15 and 16. The peak growth was observed during 18h of culture at room temperature.

Generation Time: The generation time (GT) for the isolate was calculated as 1.2 hours.

Studies with extracellular products

Protein estimation of ECP

The protein concentrations for the filtered and cellophane extracted extracellular products of different growth phases (ECP extracted at different time intervals) are presented in Table 8 and Fig. 17.

ECP (cell free extract) obtained by the membrane filtration technique showed a higher value of protein when compared to the cellophane extracted ECP. The variations in concentration of protein extracted at various phases of growth indicates that, the concentration goes on increasing with time. In the case of ECP extracted by cellophane overlay culture technique, the protein concentration of ECP of 72 h was lower. Also, it is clear that the ECP concentration of extracts from medium containing 3.5% sodium chloride was lower compared to ECP extracted with ordinary nutrient agar. The peak values of protein was obtained at 48 hours and 24 hours respectively for ECP of ordinary nutrient agar medium and medium containing 3.5% sodium chloride. The protein concentration values for ECP extracted by both methods are given in Fig. 17.

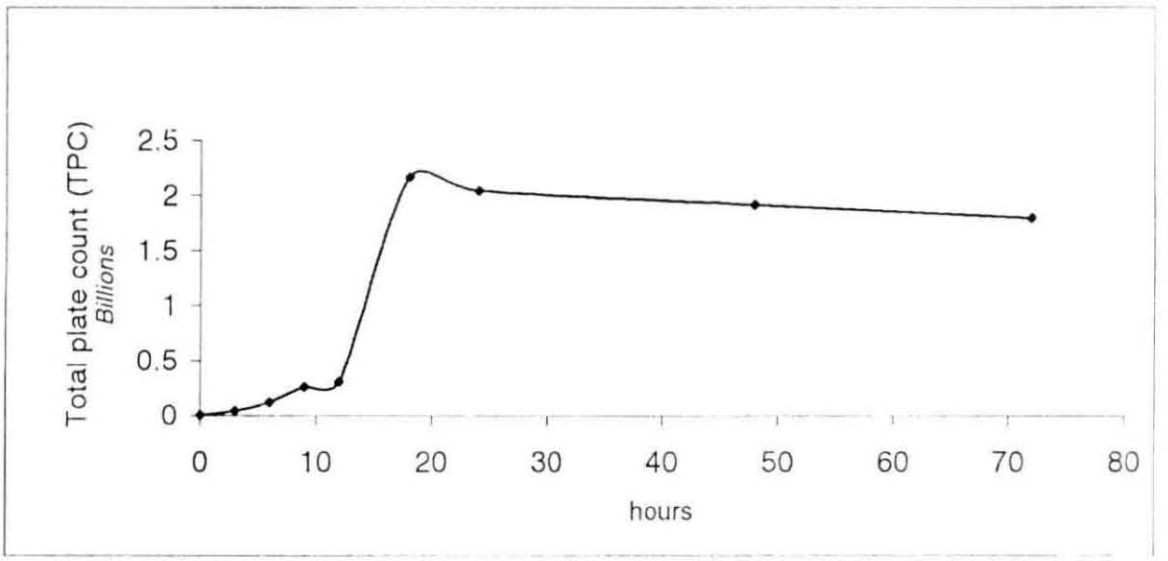


Fig. 15. Growth pattern of S. *marcescens* by the total plate count method

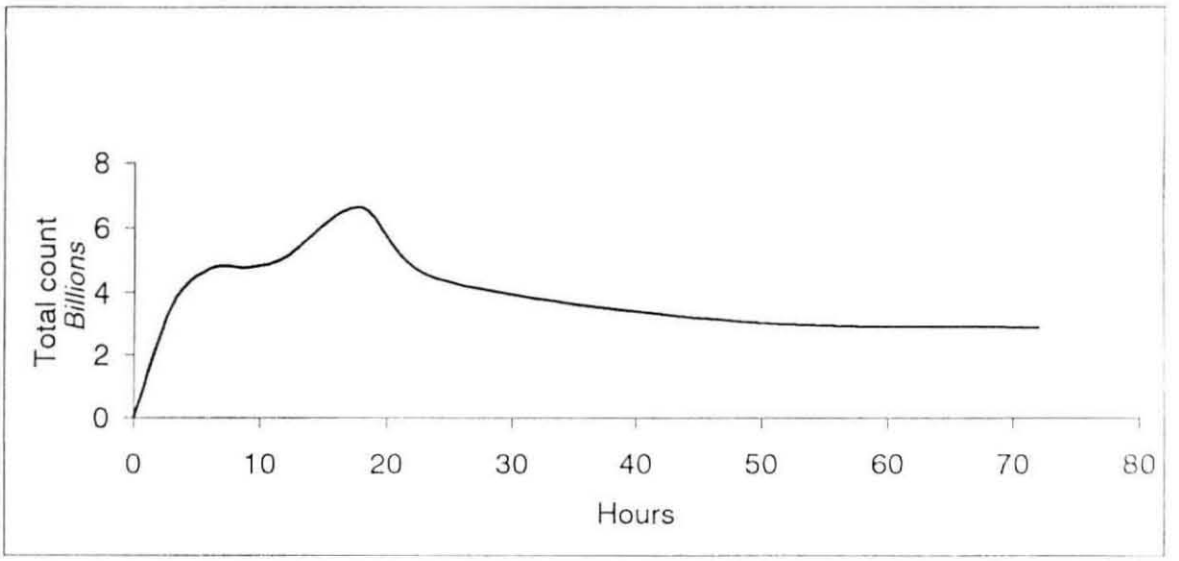


Fig. 16. Growth pattern of *S. marcescens* by the direct count method

Table 8. Protein values for ECP drawn at different phases of growth

ECP extracted at: (h)	Protein concentration(μg/ml)		
	Filtered ECP	ECP by cellophane method	
		0.5%NaCl	3.5%NaCl
0	137.2		
3	150.9	79.03	46.36
6	156.8	87.27	81.81
9	211.7	90.54	87.27
12	254.9	105.81	99.27
18	260.7	147	130.9
24	313.7	163.63	136.36
48	374.5	185.45	95.45
72	394.1	98.18	87.27

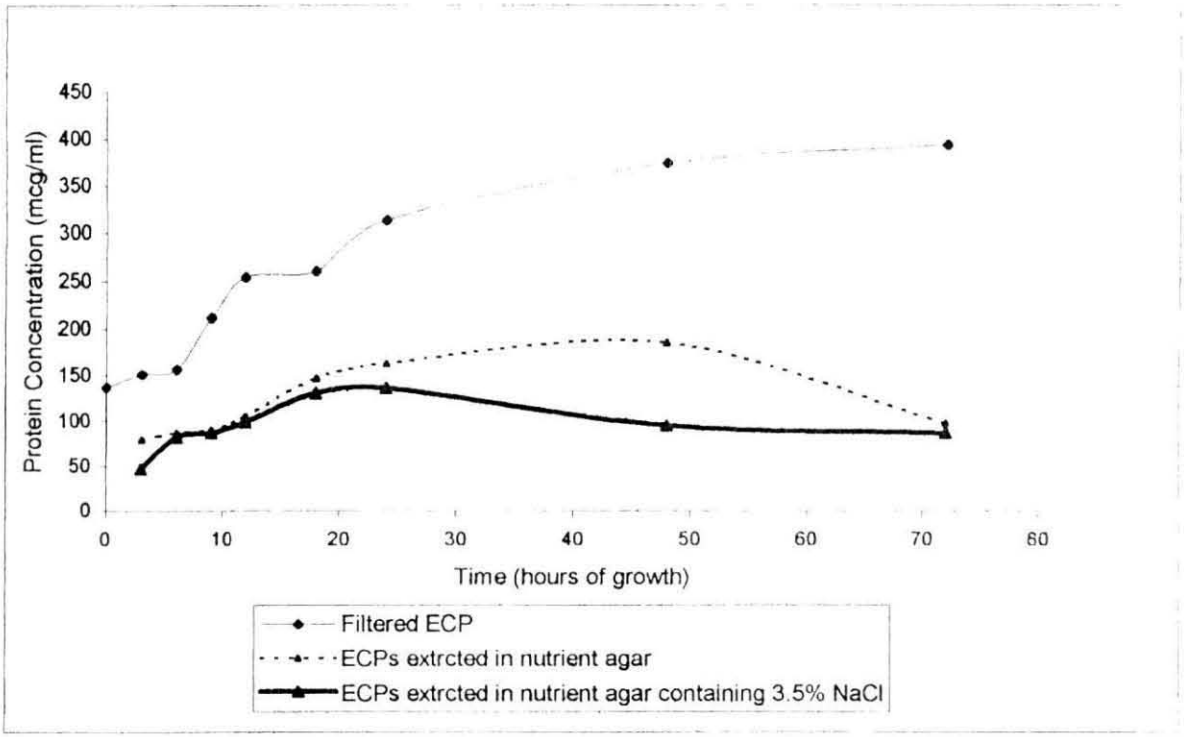


Fig. 17. Protein concentrations of extracellular products of *S. marcescens*

In vitro activities of the filtered ECP

The filtered ECP (both heat treated as well as non heat treated) was subjected to *in vitro* proteolytic activity studies. The results are given in Table 9.

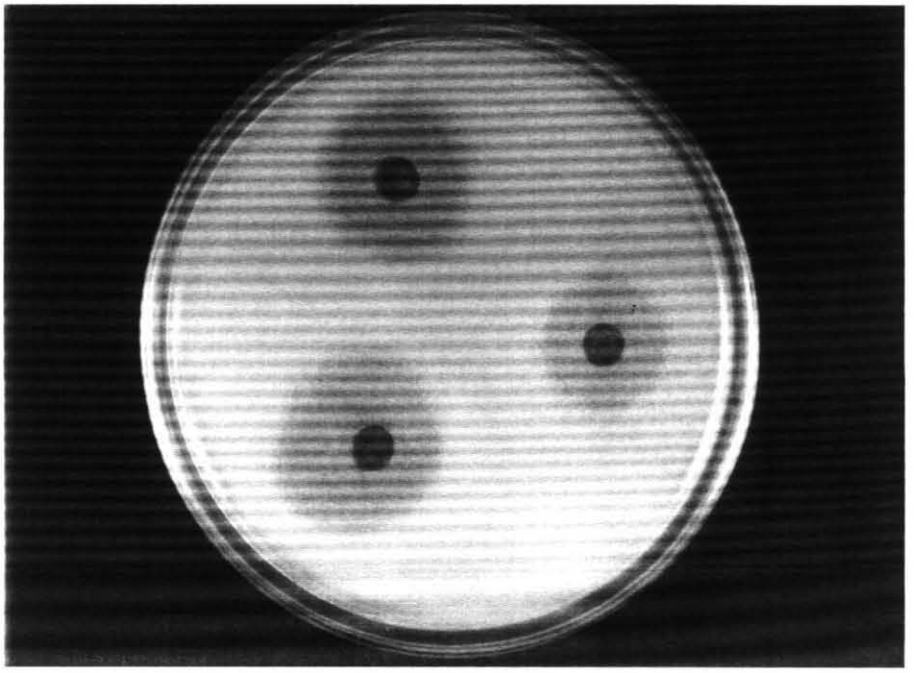
Proteolytic activity of the ECP of the bacteria was not lost on heat treatment of the ECP although diameters of zones of activity were reduced. Zones of gelatin hydrolysis were 18 mm and 24 mm dia respectively for ECPs of 6 h and 24 h culture when they were not heat treated, whereas for heat treated ECPs, extracted at the same time, clearing zones were of 16 mm and 18 mm dia, respectively. When casein was used as the substrate, the zone of clearing for ECPs extracted from 6 hour culture was 12 mm, and 18 mm for ECP from a 24 h culture, whereas the diameters were 13 and 15 mm respectively for heat treated ECPs.

It can also be seen that ECPs extracted at 24 h had high activity compared to ECPs extracted at 6 h, as indicated by the zones of hydrolysis which were 24 mm and 18 mm respectively for gelatin and casein substrates for non heat treated ECP of 24h while the diameters were 18 mm and 12 mm for the same substrates when non heat treated ECP of 6 h was used. The diameters were 18 mm and 15 mm respectively for gelatin and casein respectively when heat treated ECP of 24 h was used whereas the 6 hour heat treated ECP gave zones of clearing of diameter 16 mm and 13 mm. (Table 9; Plate 8).

Table 9. Proteolytic activity of ECP of *S. marcescens*

Substrate	Proteolytic activity zone (mm) [Non heat treated ECP]		Proteolytic activity zone (mm) [Heat treated ECP]	
	6 h	24 h	6h	24 h
Gelatin	18	24	16	18
Casein	12	18	13	15

Plate 8. Gelatin liquefaction of *S. marcescens* ECP extracted at 18 hours of growth



Pathogenicity of ECP to experimental fish

After administration of ECP by IP route, the clown fish started dying in temporal pattern within one hour of injection. The cumulative percentage mortalities of the fish in the tested ECP concentrations are given in Table 10. In the control group, there was no mortality. In the higher dose of 29.4μg, 100% mortality was noted. 75% percent mortality was noticed in 14.7μg ECP. These results indicate a dose-responsive mortality towards ECP, which could be correlated to the virulence of the isolate.

From the probit chart (Miller and Trainter, 1944), the lethal dose was estimated to be equal to 12.0226 μg/ fish (Fig. 18), which is approximately equivalent to 2.67 μg/g f fish.

The medial lethal time (LT_{50}) taken for 50% mortality was estimated to be approximately 5 hours from the graph.

Electrophoretic studies to determine the molecular weight

The banding patterns of ECPs extracted from 3, 6, 9, 15, 18, 24, 48 and 72 h cellophane overlay culture, both in ordinary nutrient agar and nutrient agar supplemented with 3.5% sodium chloride are given in Plates 9 and 10.

From the semi log graph, the molecular weights of proteins were calculated and the results showed that there was differences in expression of protein at different time intervals and also that the ECPs extracted from nutrient agar supplemented with 3.5% sodium chloride showed variation in expression of protein when compared to ECPs from normal nutrient agar.

Table 10. Cumulative percentage mortality of clown fish to different doses of ECP of *S. macrescens* isolate

Dose (µg)	Percentage mortality	
	Experiment	Control
29.4	100	0
14.7	75	0
9.8	25	0

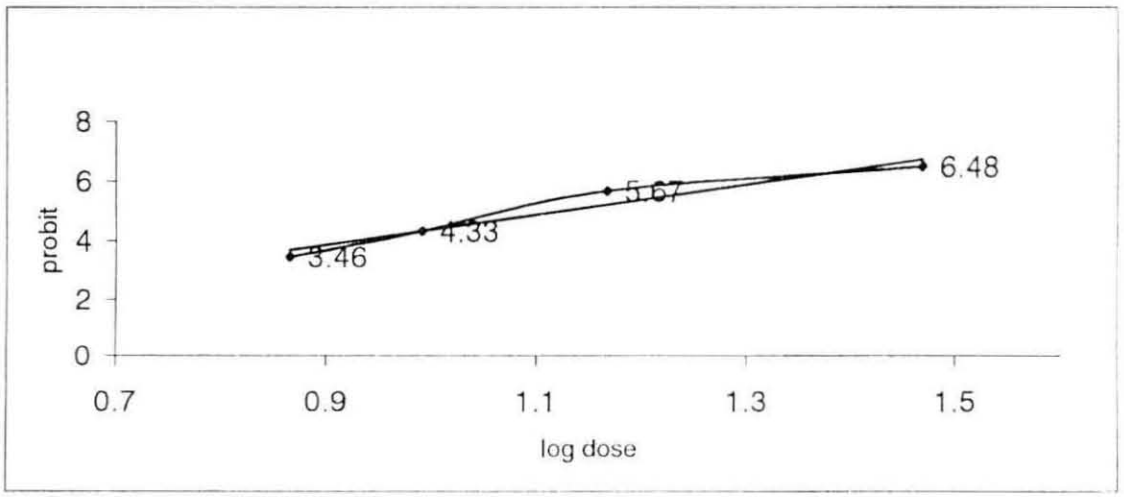


Fig. 18. Probit chart for estimation of median lethal dose of ECP

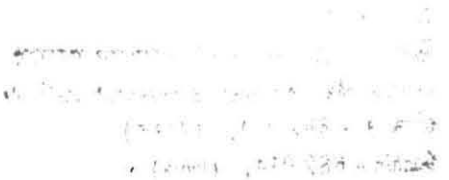


Plate 9. Protein profiles of ECPs (extracted in nutrient agar) in SDS-PAGE (Slots: 1-0 h ECP, 2 -3h ECP, 3 -6h ECP, 4-9h ECP, 5 -18h ECP, 6 -24h ECP, 7-48 h ECP, 8 - 72h ECP, 9 - standard molecular markers in kiodaltons)

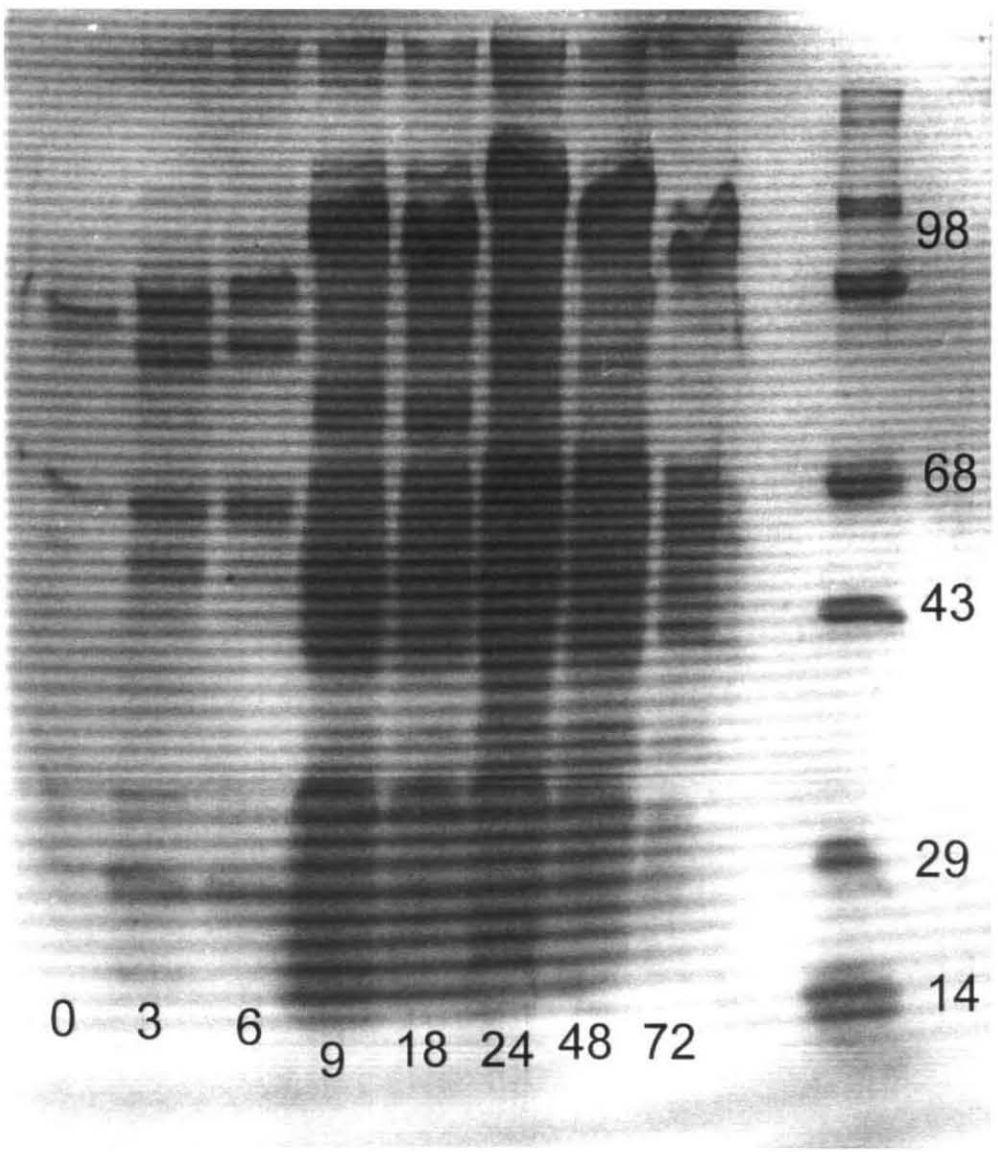
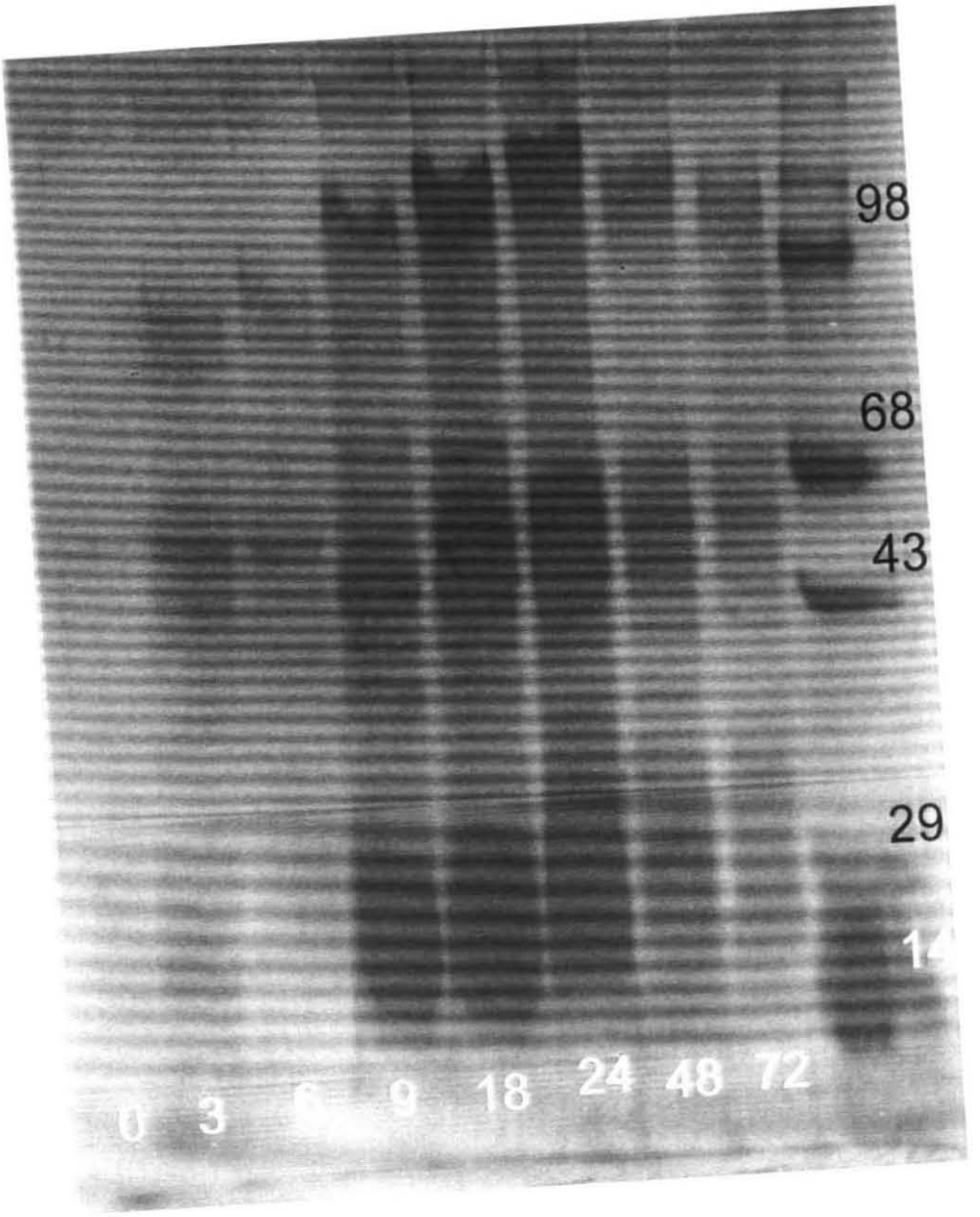

98
68
43
29
14
0
3
6
9
18
24
48
72

Plate 10. Protein profiles of ECPs (extracted in nutrient agarsupplemented with 3.5% sodium chloride) in SDS-PAGE((Slots: 1-0 h ECP, 2-3h ECP, 3-6h ECP, 4-9hECP, 5-18h ECP, 6-24h ECP, 7-48 h ECP, 8-72h ECP, 9- standard molecular markers in kiodaltons)


98
68
43
29
0
3
9
18
24
48
72

ECP from ordinary nutrient agar: It was seen that ECP of 3 h had two prominent protein bands of molecular weights, 70 and 43 kDa. At 6 h, additional proteins were expressed, especially one of molecular weight 62 kDa. Also, proteins of lower molecular weights were expressed. At 9 h, the expression of proteins was more or less similar to that of 6 h. At 15 h, expression of proteins of higher molecular weights as well as lower molecular weights became clear. But those ranging from 60 kDa to 80 kDa were found to be absent. Instead, proteins of higher molecular weights, from 92 kDa to 100 kDa appeared along with proteins of lower molecular weights up to 11 kDa. This expression was retained up to 72 h more or less in a similar way. Important characteristics were stronger expression of proteins from 15 to 48 h and the absence of proteins of molecular weight 92 kDa at 24 and 48 h, which reappeared at 72 h. It was also noticed that at 72 h, the expression of most of the proteins became feeble (Plate 9).

ECP from nutrient agar containing 3.5% sodium chloride: ECP extracted at 3 h had feeble expression of only one protein of molecular weight 41 kDa, with still feeble expression of three more bands whereas at 6 h, other than this protein, some others of lower molecular weights were expressed which were repeated at 9 h. From 15 h onwards, proteins of higher molecular weights (100 kDa) and those of 40 kDa to 50 kDa started appearing. Also at this time, expression of proteins of lower molecular weights became strong which became weaker at 48 h and 72 h. At 24 h, the expression of all the major proteins were found to be strong (Plate 10).

DISCUSSION

It is evident from the earlier two chapters of the thesis that bacterial diseases are responsible for high mortality among the aquarium held marine ornamentals. In this chapter experiments were carried out to determine the infectivity as well as virulence of one of the important isolate, *S. marcescens* by using live cells as well as the cell free culture filtrate.

The *Serratia* genus is reported as pathogen of rainbow trout (Nieto *et al.*, 1990), brook trout (Llewellyn, 1980) Atlantic salmon (McIntosh and Austin, 1990; Llewellyn, 1980), turbot (Vigneulle and Baudin Laurencin, 1995) and white perch (Baya *et al.*, 1992) whereas this species is reported as the pathogen of marine ornamental fishes for the first time in this thesis. Certain species belonging to this genus are reported as potential pathogens. According to studies by Baya *et al.* (1992), *S. marcescens* is a species of pathogenic potential, whose pathogenicity mechanism is determined by the proteolytic enzymes, which were toxic to fish and homoeothermic cell lines. McIntosh and Austin (1990), described the species, *S. liquefaciens* as an etiological agent of proteolytic disease in Atlantic salmon, *Salmo salar*, in Scotland and reported that pathogenicity mechanism could be correlated to the production of proteolytic extracellular products. The other species associated with fish diseases was *S. plymuthica* (Nieto *et al.*, 1990).

Knowledge concerning the pathogenesis of many bacterial diseases in marine fish is limited, especially for those diseases, which occur among the warm water species. Studies on bacterial pathogens in marine ornamental fishes is also limited, except most on infection of pomacentrid eggs with *Pseudoalteromonas piscicida* (Nelson and Ghiorse, 1999), *V. damsela* infection of damselfishes (Love *et al.*, 1981).

Pathogenicity of live cells

The results of the experimental infectivity studies using live bacterial cells indicated that the mortality of fish followed a dose-dependent pattern. At 10^6 cells it exceeded 50% mortality while at 10^5 cells, the mortality was 20% only, as could be noted from Table 6. Based on the probit determination, the LD_{50} value was determined as 5.01×10^5 cells (Fig 14).

The number of cells required to bring about the lethal effect is slightly higher than *A. salmonicida* for salmonids. Being a primary pathogen, *A.salmonicida* could have caused mortality among the experimental group of salmonids, at LD_{50} values ranging from 50 cells to 1×10^3 cells as demonstrated by Olivier *et al.* (1990) and Gudmundsdottir *et al.* (1997). Khalil and Mansour (1997) found that *A. hydrophila* was found to produce haemolytic and proteolytic exotoxin, lethal to tilapia with LD_{50} value of 2.1×10^4 cells/fish as well as heat stable unknown virulent factors that were responsible for 20% mortality.

Other pathogenic isolates of fish including *Pseudomonas aeruginosa* and *A. hydrophila* were tested for their pathogenicity (Lipton, 1987). It was observed that the fish isolate of *P. aerurginosa* had a lethal dose of 1.5×10^{-5} cells per fish for *Cyprinus carpio* and 4.2×10^5 cells for *Oreochromis mossambicus*. The fish pathogen *A. hydrophila* had lethal doses of 2.1×10^6, 6.8×10^5 and 3.2×10^6 respectively for *C. carpio*, *Labeo rohita* and *O. mossambicus* (Lipton, 1987).

In the case of *E. ictaluri* the pathological studies revealed that the bacteria exhibited the characteristic symptoms of the disease upon exposure to 5×10^8 cells (Newton *et al.*, 1989). However, in their experiment, the infections were carried out using immersion exposure of the pathogen. Similarly, ulcerations in damselfish, *Chromis punctipinnis* by *V. damsela* required larger number of cells ranging from (10^7 to 10^8 cells) using swab treatment exposure (Love *et al.*, 1981). The requirement of cells to produce infectivity was lesser

when experiments were carried out by intramuscular or intraperitoneal injections. For example, *S. marcescens* required an LD_{50} value of 5×10^3 cells to 5×10^5 cells for rainbow trout and striped bass respectively by intraperitoneal or intramuscular injection (Baya *et al.*, 1992). Plumb and Sanchez (1983) reported 100% mortality among channel catfish injected intraperitoneally with 1.5×10^3 cells of *E. ictaluri*. The same authors have indicated the susceptibility of different species of experimental fish such as Tilapia, golden shiner, largemouth bass and bighead carp to experimental infections with *E. ictaluri*.

Although different experimental fish were not tried, in the present investigations, the susceptibility of *A. sebae* to *S. marcescens* was found to be comparable to most of the bacterial pathogens reported earlier. In the present study, intraperitoneal route was tried as the route of entry of live cells and the LD_{50} value obtained in the study, corresponded to the results above. It was also noticed that the lethal dose required was lower than what was required by *V. damsela* and *E. ictaluri* as indicated earlier from the studies of Newton *et al.* (1989) and Love *et al.* (1981).

The results of pathogenicity experiments of strain *S. liquefaciens* K2, by McIntosh and Austin (1990) using Atlantic salmon as experimental group resulted in proteolytic diseases with 10^3 bacterial cells per fish within 72 hours. The same authors reported that 100% mortality was achieved within 24 hours by administering 10^4 cells in the case of the same host. When injected, the strain caused pronounced damage to the musculature at the site of injection. Parallel experiments with rainbow trout revealed that intramuscular injection of 10^7 cells per ml caused similar changes while intraperitoneal injection led to less severe changes.

Vigneulle and Baudin Laurencin (1995) recorded that when injected with *S. liquefaciens*, which is known to be highly proteolytic in rainbow trout, 0.1ml bacterial suspension in 0.9% w/v saline containing 2.7×10^7 cells, resulted in 12.5% mortality. In the same study, the mortality percentage was

reduced to 8.0% when the cells were slightly reduced to 1.1×10^5. All the dead fish displayed internal hemorrhages. However, these authors could not record mortality among turbot *Scophthalmus maximus* although a high inoculum of 1.3×10^6 cells were administered to experimental fish. External or internal lesions of fish were also not recorded, despite the fact that, initially *S. liquefaciens* was isolated from turbot, cultured in marine cages, in which clinical signs like hyper pigmentation of skin, lesions of kidney and spleen, leading to a low but continuous mortality were recorded in the case study.

The red-pigmented species, *S. plymuthica*, repeatedly isolated from moribund rainbow trout fingerlings had a lethal dose of 10^5 cells, when administered as intraperitoneal injections into rainbow trout. The affected fish showed signs of tissue damage due to challenge (Nieto *et al.*, 1990). Studies by Austin and Stobie (1992) with *S. plymuthica* indicated total mortality of fish within 7 days on experimental infection with 10^4 cells via intraperitoneal or intramuscular route.

Regardless of the inoculation routes, the white perch isolate (RB 469) of *S. marcescens* was reported to be pathogenic to striped bass with a mean lethal dose of 1×10^5 cells (Baya *et al.*, 1992). In the present study, intraperitoneal injection with 5.01×10^5 cells of *S. marcescens* isolate in clown fish led to 50% mortality. In the above case studies by Baya *et al.* (1992) and Nieto *et al.* (1990), cell concentrations of 10^5 were required for the median lethal dose, which indicate similar findings among *A. sebae* using *S. marcescens*.

The inoculation studies of Llewellyn (1980) with 0.2 to 0.3ml suspensions of isolate showing similarities to *S. liquefaciens* in brook trout and landlocked Atlantic salmon proved that signs of disease occurred after 2 to 7 days of inoculation. But, the number of cells required for pathological signs was not reported.

From the above studies with pathogenic isolates *Serratia* isolates it is now clear that the infective dose of live cells required for experimental fish are almost similar and are comparable to that of other known pathogens. The virulence studies of Baya *et al.* (1992) carried out with the same species, *S. marcescens* on rainbow trout and striped bass, the studies on pathogenicity by Vigneulle and Baudin Laurencin (1995) on rainbow trout, the studies of McIntosh and Austin (1990) on Atlantic salmon and inoculation studies of Llewellyn (1980) with *S. liquefaciens* along with observations of Nieto *et al.* (1990) on the isolation and virulence of *S. plymuthica* clearly shows that most of the species belonging to the *Serratia* genus should be considered as potential pathogens.

Influence of the ECPs of Pathogenic Isolates on Experimental Group of Fishes

The results of the ECP on infectivity studies indicate that the experimental group of fishes succumbed to the high dose of 29.4µg as could be seen from Table 10 and Fig. 18. Less than half of the dose was found to be sufficient to kill more than 50% of the fishes. From the probit chart (Fig. 18) as well as from the cumulative percentage mortality, the LD_{50} value was determined as 12.02 µg per fish (approximately 2.67µg/g). The median lethal time was estimated as five hours, which indicated that the ECP produced by the bacteria was a very potent toxin. Considering the *in vitro* proteolytic activity, the ECP at 18h and 24h of *S. marcescens* could be regarded as potential pathogenic ones although the proteolyic activity started appearing from 6h onwards

These above results are also comparable with the studies of other authors. The LD_{50} value of ECP of the three strains of *S. marcescens* in fingerling rainbow trout (4g) and striped bass (7g) was found to range between 0.22 and 4.8 µg protein per gram of fish (Baya *et al.*, 1992). The value obtained in the present study for *A. sebae* with *S. marcescens* was almost half of this value. Baya *et al.* (1992) has recorded that 4.8 µg protein /g was required for striped bass whereas rainbow trout succumbed at the lethal dose of 0.4µg/ g and have declared that these values were comparable to those reported for *Aeromonas* and *Vibrio* species.

Baya *et al.* (1992) have also reported that when undiluted ECP of *S. marcescens* was used for injecting fish, mortality occurred within one hour. In the present study also, the fishes started dying within one hour of injection, although the median lethal time required for mortality was found to be 5 hours. From the studies of McIntosh and Austin (1990), it was seen that injection of ECP containing 0.12 mg protein of the K2 strain of *S. liquefaciens* per ml into Atlantic salmon via intramuscular route resulted in death within 48 hours and had symptoms similar to that of infection with live cells.

In the case of *A.salmonicida*, causing furunculosis, Munro (1982), postulated that pathogenicity was associated with the capacity of the pathogen's extracellular virulence factors, based on his earlier observations that the highly proteolytic ECP of *A.salmonicida* was lethal to rainbow trout. Sub lethal doses of the same preparations resulted in producing the furunculosis syndrome, when administered intraperitoneally or intramuscularly. Tajima *et al.* (1983) demonstrated that LD_{50} value of ECP of *A. salmonicida* was 2.4 µg /g of fish while in the case of *A. hydrophila* even a low lethal dose such as 150 ng/g (Rodriguez *et al.*, 1992) was also reported. Thune *et al.* (1982) stated that the LD_{50} value of crude ECP of *A. hydrophila* was 18 µg protein per gram for channel catfish in the case of heat labile protease while another heat stable fraction had a LD_{50} value of 3.0 µg/g.

Fyfe *et al.* (1986) observed that hemorrhages and necrosis were observed after injection with 0.8 µg of protein of *A. salmonicida* in Atlantic salmon. These authors related the protease activity of the protein which corresponded to the *in vitro* output of 3×10^7 cells of *A. salmonicida*. In the present study, the ECP used for *in vivo* studies was extracted from 18 h of growth of the isolate which had concentration of 147 µg protein/ml corresponding to 2.16×10^9 cells, i.e., 1.47×10^7 cells were required for production of 1 µg protein/ml.

From studies conducted by Lipton (1987), ECPs of *P. aeruginosa* and *A. hydrophila* were found to be lethal to fish with LD_{50} values of 12.95µg/g and 18.31µg/g in the experimental host, *O. mossambicus*. In the same study, it was noticed that the activity of ECP was increased at log phase of growth, whereas in the case of *A. hydrophila* the activity was increased at stationary phase. In the present study with *S. marcescens*, even though the maximum growth of bacteria was observed at 18h, the ECP production in terms of protein concentration was at it its maximum at 24 h when ECP was obtained by the cellophane method with media containing 3.5% sodium chloride while, with ordinary nutrient agar extracted ECP had higher protein values at 48 hours.

The *Vibrio* group of fish pathogens produces ECPs, which had hemolytic, cytotoxic and hemagglutinating activities, which could be related to the virulence of the pathogen. Inamura *et al.* (1984) determined that the lethal dose of heat labile (70 ^{0}C, 10 minutes) crude ECP of *V. anguillarum* from a highly virulent strain was 15µg/ g of goldfish. These authors have also reported that the proteolytic and lethal activities of *V. anguillarum* were higher among the virulent strains than the low virulent strains. The same authors in 1985, reported that the purified protease of *V. anguillarum* had an LD_{50} value of 1.7 µg protein/g weight for goldfish.

Similarly, Santos *et al.* (1991) recorded that the ECP from *V. anguillarum* strains of serotypes 01 and 02 exhibited lethal dose for turbot, salmon, and trout ranging from 4.52 to 7.32 µg protein/g fish. From another study conducted by Lee (1995), the ECPs of a pathogenic strain of *V. anguillarum* S3y were found lethal to fish with a minimum lethal dose of 0.52 µg/g fish body weight. A 34 kDa protease was purified from the ECP of S3y strain and was demonstrated to be a toxin by intraperitoneal injection in the grouper, *Epinephelus malabaricus*.

In a study conducted by Fouz *et al.* (1993), it was observed that the ECP from several isolates of *V. damsela* were strongly lethal for fish with LD_{50}

values ranging from 0.06 to 3.7 µg protein/g fish. Virulence of *V. furnissii* isolates for eels was demonstrated by intraperitoneal injection of living cells (Esteve *et al.*, 1995) with LD_{50} value of 10^6 cfu/fish. The same authors reported that ECPs produced by *V. furnissii* displayed protease and haemolytic activities and were also lethal to elvers, which induced pathological signs, proving their role in the pathogenicity for fish.

In vitro and *in vivo* activities of culture filtrate, extracellular product (ECP), protease, hemolysin, crude LPS, pure LPS and sonicated cell-free supernatant of *F. maritimus* were examined for their pathogenicity to black sea bream and red sea bream fry, by Baxa *et al.* (1988). They observed that median lethal doses of the culture filtrate and extracellular product (ECP) of *F. maritimus* following intraperitoneal injection in black sea bream were 123 and 25.5 µg/g respectively and that black sea bream required lower LD_{50} values than red sea bream.

Thus, the results of toxicity of ECP obtained in the present study are comparable to those of many of the exotoxins or ECPs already reported by the earlier authors. It could be observed that the concentration of the ECP protein required for bringing about lethality was similar to those of other well known pathogens. However, the activity of the different specific proteins of ECP was not characterised.

In the artificial infection studies, a reddish colour was observed in the ventral portion near the area of injection as well as in the opercular region in many of the injected fish (Plate 6). Similar observations were recorded by Baya *et al.* (1992) in white perch on injection with *S. marcescens* isolate, in which case, the injected fish acquired a reddish colour along the lateral line and head region which is considered due to the multiplication of the bacterium and the concomitant synthesis of prodigiosin pigment. Nieto *et al.* (1990) also had recorded that during experimental infection of rainbow trout with *S. plymuthica*, external discoloration along with abdominal swellings, was noted. However, in

contrast, in natural infections, the fish did not display external clinical signs, which led them to suggest that the experimental conditions need not reproduce development of natural infection.

As per the observations by Baya *et al.* (1992) some of the dead striped bass showed strong signs of muscular tissues, slight signs of haemorrhgic septicemia. In the experiment with clown fish also, more than 50% of the experimental group exhibited fin and tail rot in addition to exophthalmic conditions.

Stability of ECP to temperature

In the present experiment, the *in vitro* assay (Table 9) indicated that the proteolytic activity of ECP were reduced to almost to two third which when heated to 50^{0}C for 10 minutes, which indicated the possible thermolabile nature of the ECP. As the ECP was found to be thermolabile, the further experiments on fish pathogenicity with heat-treated ECP were not carried out in the present investigations.

Baya *et al.* (1992) found that the ECP produced by *S. marcescens* was thermolabile and its toxicity was totally lost on heating at 100^{0}C for 10 minutes.

In most of the ECP studies of fish pathogenic bacteria, several authors have reported the thermolabile characteristics of the ECPs. Inamura *et al.* (1984) also studied a heat labile exotoxin produced by *V. anguillarum* and indicated that the exotoxin of *V. anguillarum* which was toxic to gold fish, Japanese eel, Ayu and mouse was heat labile when heated at 70^{0}C for 10 minutes. In the study cited earlier by Santos *et al.* (1991), it was noticed that all the biological activities of ECP of *V. anguillarum* strains of serotypes 01 and 02, were lost after heat treatment at 80 ^{0}C for 10 minutes. Santos *et al.* (1992) reported that although, most of the ECP isolates of *A. hydrophila* and *V. anguillarum* with different degrees of virulence for fish, displayed amylolytic,

proteolytic, haemolytic, cytotoxic and dermatotoxic activities, all such activities were reduced after heating at 56 ^{0} C for 30 minutes and totally lost by heating at 80 ^{0}C for 10 minutes.

Fouz *et al.* (1993) reported that most of the activities of ECPs of several isolates of *V. damsela* are associated with exoenzymes and their *in vivo* and *in vitro* biological activities were considerably reduced after heat treatment at 100 ^{0}C for 10 minutes, although not totally lost in the highly virulent strains.

Khalil and Mansour (1997) observed that complete inactivation of the protease enzyme from *A. hydrophila* occurred after heating the ECP at 80^0 C for 10 minutes or 70 ^{0}C for 15 minutes. As indicated earlier, they have found that *A. hydrophila* produced haemolytic and proteolytic exotoxin lethal to tilapia with LD_{50} value of 2.1 × 10^4 cells/fish as well as heat stable unknown virulent factors that were responsible for 20% mortality. The lethality of ECP was decreased by heating and completely lost by boiling at 100 ^{0} C for 10 minutes.

Protein concentration of ECP

It was seen that the maximum production of protein was observed from 18 to 48 hours of growth of the bacteria (Table 8; Fig. 17). In the case of filtered ECP, the protein values continued to increase even after 48 hours, which could be due to the presence of media components in the culture. In the case of ECP extracted by cellophane method, with the ordinary nutrient agar medium, the maximum values of protein concentrations were recorded during 48 h of growth, however, since the *in vitro* studies showed that the ECP extracted at 18h had the maximum activity, it was selected for *in vivo* studies. In the case of media containing 3.5% NaCl, the protein values were fond to be maximum at 24h which ruled out the possibility of production of more proteins with addition of sodium chloride in the medium.

Growth requirements

The results of the experiments to detect the expression of ECP at different growth phases indicated that at 24 hours the activity of ECP was high compared to 6 hours, which indicated that,the cell growth as well as the media component plays a vital role in determining the production of ECP. In addition, the ECP production was also determined based on the virulence pattern of the isolate. According to Stevenson and Allan (1981) the optimal production of hemolysins and protease by *A. hydrophila* was noted with a complex carbon and nitrogen source medium. However, both activities were significantly reduced when amino acids were replaced by glucose and ammonium sulphate in the medium.

Many of the bacteria produce proteolytic enzymes during the course of infection and pathogenesis (Ellis and Grisley, 1985). By incorporating calcium at the rate of 5.4 to 21.6 µg/ ml along with L.histidine (1g/L) supplemented medium was found to produce maximum level of hemolysin and protease by *A. hydrophila* (Riddle *et al.*, 1981). Wodzinski (1979) indicated that the yields of exocellular enzymes could be enhanced to greater levels by improving the culture conditions and techniques.

Although in the present study, nutritional factors responsible for production of ECPs were not investigated, the experimental studies proved that the production of ECP varied at different phases of growth as indicated by the PAGE studies. Media components could have played a significant role in the growth of bacteria. It was also noticed that production of ECP showed variation with the addition of sodium chloride at the rate of 3.5% to the nutrient agar medium. Although, the pattern of expression was more or less same in both media (Plate 9 and 10), the isolation of protein of molecular weights ranging from 20 to 50 kDa into more number of individual bands occurred in media in which 3.5% sodium chloride was added. This could be possibly due to the fact that isolate was obtained from marine fish.

Molecular weight of ECP of *S. marcescens*

Since the ECP protein characterisation of fish pathogenic *Serratia* were not carried out earlier, comparison of the protein bands with other species of *Serratia* could not be made. However, based on studies on other fish pathogenic bacteria, the protein profiles obtained in the present study could be evaluated.

In the present investigations, the expression of protein with molecular weight 62 to 70 kDa intensified from 15 hours of culture, which corresponded to about 10^9 cells. The intensity of protein bands occurring at this period corresponded to the molecular weights obtained for ECPs of other pathogenic isolates. For example, Tajima *et al.* (1983) found out that the proteases of *A. salmonicida* was isolated as a component with a molecular weight of 70kDa. It was also observed in the present studies that, from 6h onwards, major bands started which were retained up to 48 h indicating that these proteins played a major role in pathogenicity.

Studies conducted by Chen *et al.* (1997) with ECPs of *Mycobacterium* spp. isolated from ornamental fish had major bands at 65 and <14 kDa.

Proteases with molecular weights of 47, 40, 34 and 32 kDa were identified in ECPs of *F. columnaris* by using substrate SDS-PAGE and similar protease profiles were associated with ECP collected at intervals during several days in culture (Bertolini and Rohovec, 1992). In the present study also, studies were carried out with ECPs collected at intervals of 3 hours and protein profiles obtained showed that similar types of proteins fractions were obtained at different phases of growth although their intensity varied with time. For the pathogenicity studies, the ECPs drawn from 18 h growth phase were consistently taken, which indirectly indicate that both higher and lower proteins

synergistically could have resulted in the enzymatic activity of the ECP and thus resulted *in vivo* pathogenicity.

In the case of *Aeromonas* exocellular products irrespective of the species and serogroups, they possessed major component with molecular masses ranging between 55 and 28 kDa (Santos *et al.*, 1996). In the present study with *S. marcescens*, majority of the proteins belonged to proteins of such intermediate molecular weights. Most of them had molecular weights from 20 kDa to 70 kDa.

Lee (1995) purified the 34 kDa protease from ECP of a pathogenic strain of *V. anguillarum* S3y, which was demonstrated to be a toxin by intraperitoneal injection in grouper, *E. malabaricus*..

The electrophoretic (SDS-PAGE) analysis of ECP of different field strains of *Pasteurella piscicida* showed evidence of only quantitative differences inside the panel of strains and between the same strain, collected at different *in vitro* cultivation times (Mazzolini *et al.*, 1998). This observation proved that though the presence of proteins of molecular weights at different periods of growth of the bacteria may indicate different types of protein, it need not be different qualitatively, which was evident from the present study also, where the proteins of molecular weights varying from 11 kDa to 100 kDa were identified which had varying protein concentrations indicating that the proteins varied quantitatively. The nature of protein or qualitative analysis of the proteins needs to be carried out after purification of different fractions, which were not carried out in the present study.

It can be noted from the investigations that the genus *Serratia* was involved in the disease conditions of economically important marine ornamentals and was a pathogen having potential to cause havoc under stress conditions both in marine and fresh water fish. This pathogen needs attention as a potential member of Enterobacteriaceae family whose pathogenic significance

was proved from contemporary studies. Factors of virulence due to cell surface structures, exocellular products, serotyping of the various strains of the pathogen etc. need further attention, in addition to the cataloguing of the taxonomic status of various species, sub species and strains of *Serratia*.

CHAPTER 4

MANAGEMENT OF BACTERIAL DISEASES USING ANTIBACTERIAL COMPOUNDS (SYNTHETIC AND NATURAL) AND AUTOGENOUS VACCINE

Maintenance of fish in healthy conditions is to be accomplished by careful husbandry, for which disease management is a vital strategy. Diseases due to various pathological agents require different types of treatment, based on the aetiology of the pathogen and the effect on the host organism. The management of bacterial disease has been a practice, which was being carried out for a long period of time. Antibiotics as wells as other antimicrobial agents are used as chemotherapeutic measures in aquaculture to treat many types of infections. The use of these compounds has been considered a sensitive issue of late, which led to social problems due to the possible effects of pollutions in water bodies. Because of the same reasons, their use has been discouraged or confined to the minimum possible varieties, which are less harmful to the ecosystem.

The alternative is the biological control of diseases and immunoprophylaxis. In addition to the use of antimicrobial agents as chemotherapeutic measures in aquaculture, immunisation strategies and other immunostimulants are being practiced as management measures along with biological methods of control. As far as marine ornamental fish are concerned, the control of disease was practiced mostly via chemotherapy using antimicrobial compounds. Very little information is available on experiments on immunisation, effective use of probiotics, biological control and marine natural products to prevent disease occurrence in the marine ornamental fish. In the present study, observations on management of bacterial diseases were carried out by using chemotherapeutants as well as by extracts of seaweed and sponges containing marine bioactive compounds along with immunisation of fish by formalin killed cells. It was observed that the pathogenic bacteria encountered in the marine aquaria could be effectively controlled by methods of chemotherapy, biological control and immunoprophylactic measures.

Chemotherapy in Aquaculture

With the increasing importance of farming of different candidate species for aquaculture, infectious diseases have become very common. In spite of the studies on vaccines and other immunoprophylactic measures, chemotherapy is still essential in controlling diseases (Austin *et al.*, 1981) and is likely to remain as such in the near future too. The limitations of available effective bacterins and general methods of disease control made chemotherapy an effective and flexible method of disease management. Coinciding with these, studies on therapeutics and their residues in water and tissues were also carried out (Ishida, 1992).

Use of drugs in aquaculture and aquarium keeping has been in practice for a long time and their use in confined systems such as aquariums and fish culture experiments followed the use of such drugs as practiced in human medicine (Austin, 1985). The use of antimicrobial compounds in fisheries started with the work of Gutsell (1946), who recognised the potential of sulphonamides in combating furunculosis. Austin (1984) has also reported the widespread use sulpha drugs in fish culture. Sulphonamides remained for a long time, the main stay of life saving drugs in aquaculture, especially with the identification of sulphadiazine as an effective treatment for bacterial kidney disease (Rucker *et al.*, 1951) and then with potentiation of sulphonamides, i.e. a mixture of trimethoprim and sulphonamides which have been found to be useful for the treatment of furunculosis. The effectiveness of potentiated sulphonamides for control of fish disease especially, furunculosis is given by McCarthy *et al.* (1974).

Since then, a vast range of compounds have been used in fisheries against various microbial infections, including oxytetracycline (OTC) (Sniezko and Griffin, 1951), chloramphenicol, kanamycin (Conroy, 1961), nifurprazine (Shiraki, *et al.*, 1970), oxolinic acid (Endo *et al.*, 1973), flumequin (Michael *et al.*, 1980) and many others belonging to beta lactam groups, aminoglycosides, glycopeptides, macrolides, quinolones, other potentiated

sulphonamides, quaternary ammonium compounds etc. Commonly used antibiotics include OTC, chloramphenicol, chlortetracycline, the latter being a widely applied bath treatment for aquarium fish. In recent years, the antimicrobial compounds such as oxytetracycline, oxolinic acid, flumequine, florfenicol and trimethoprim/sulpahadiazine were used in Norwegian aquaculture (Torkildsen *et al.*, 2000).

Nitrofurans were released for fish in 1970, which are able to combat systemic infections due to *Aeromonas* and *Vibrio* forms and against columnaris as nifurpirinol in Japan (Amend and Ross, 1970) and as nifurprazine in Germany (Shiraki *et al.*, 1970). Furazolidone (earlier nitrofuran) was effective in oral administration (Sniezko, 1978). The studies conducted in 70's and 80's were pioneering ones regarding the use of chemotherapeutic drugs in aquaculture. Medications were available for most of the infections caused by pathogenic bacteria and some of broad-spectrum antibiotics were used very commonly in aquaculture to control disease outbreaks. The majority belongs to beta lactams, amino glycosides, tetracyclines, sulphonamides, potentiated sulphonamides, nitrofurans, quinolones and non-classifiable groups by the fish farming industry of the EEC (Leong, 1993). However, recently the Marine Products Exports Development Authority (MPEDA), India, has banned the use of chloramphenicol, furazolidone, neomycin, nalidixic acid, sulphamethoxazole, in shrimp aquaculture (Anon, 2001).

Abuse of antibiotics

As several chemotherapeutic compounds are being introduced, it is necessary to pay attention to the products and their effects in the system, though it is difficult to compare the value of application of one drug with the other. The main factors of concern, while using the antibiotics are issues of environmental pollution and development of resistant strains of microflora. The latter becomes a hazardous problem especially if the medications are allowed to enter into the waterways, which may lead to development of resistant microflora, which finally transfer the resistance via plasmids to pathogens of

veterinary or human importance (Austin, 1985). The hazards arising due to the use of chemotherapeutants in treating fish disease have been highlighted in recent years together with increasing public awareness of their potential hazards to public health and in the case of so many, it has become a need to evaluate alternative therapeutic measures.

Considering these factors, it is essential to take care of the use and licensing of drugs in aquaculture, by the concerned authorities (Leong, 1993). In the United States, the FDA has given list of chemotherapeutants, which may be used in aquaculture and all the drugs for use in aquaculture are registred with FDA (Schnick, 1988); the use of compounds classified into GRA's (generally regarded as safe) and categories allowed in aquaculture are strictly followed to prevent the problems arising through abuse of antibiotics. Among sulphonamides, FDA cleared sulphamerazine, sulphamethazine, and sulphadiazole for use in fish diseases (Sniezko, 1978). In Europe, the EEC has a restrictive policy in the use of drugs. But in contrast to the strict control enforcements by the members of the EEC, non-EEC European countries and Japan tend to use a large variety of drugs in fish farming (Leong, 1993).

As early as during 1978, Sniezko cautioned that the use of many medically important compounds in fisheries should be discouraged. There are many advocations where compounds and drugs are excluded from use in fisheries, based on applications in human medicine, which are meant for infections of humans (Austin, 1985). Based on different studies Austin (1985), listed 30 compounds for use in aquaculture.

The norms to be followed while using antibiotics in aquaculture vary with situations. Aspects like availability of the chemicals, dosage, storage, shelf life, safety, withdrawal period, development of resistant microflora, presence of residues, environmental impact, effectiveness of the drug on surveillance etc are given importance (Leong, 1993; Austin and Austin, 1987).

Procedures for antibiotic susceptibility tests

It is necessary to determine the potency of any drug or bacterial susceptibility before it is recommended for use in aquaculture. According to Schnick (2001), international harmonisation of antimicrobial sensitivity methodologies used on aquaculture drugs is needed because of the concern for the health of cultured animals, an increase in global trade, and concern for disease resistance developing from the use of these drugs and has also stated that currently, the antimicrobial sensitivity methodologies for drugs used in aquaculture are not standardised and that situation has provided for a variety of test results worldwide.

Antibacterial susceptibility tests may be performed by dilution or diffusion methods (Finegold and Martin, 1982). The inhibitions of growth of sensitive microorganisms with selected concentrations of the antibiotics can be compared with other substances, whose ability has been determined with respect to the corresponding international standard. Fleming (1929) was the first one to record an antimicrobial susceptibility test for *Staphylococcus aureus* and penicillin.

A few procedures utilising the ability of an antimicrobial agent to diffuse through agar and inhibit the growth of the test organism on the agar medium giving a clear area or zone of no growth were described in the early days of development. Most variations in the procedure were the methods of application of antimicrobial agents; such as cutting wells into the agar (Reddish, 1929) and cylinder plate (Abraham *et al.*, 1941). In 1944 Vincent and Vincent used filter paper discs impregnated with penicillin and in 1945, Mohs described the radial streak method using 15mm discs and the use of a sensitive control organism. Other disc diffusion methods were also described at this time. Paper discs of 5 to 6mm diameter generally used today were described by Bondi *et al.* (1947). The use of tablets instead of filter paper discs was first described by Hoyt and Levine (1947). Another method of agar disc diffusion most commonly

used is that of Bauer *et al.* (1966) for testing the sensitivity of bacterial strains to standard antibiotics.

In the early years of antimicrobial susceptibility testing, there was no standardisation between the methods or even between laboratories. This problem was recognised by Gould and Bowie (1952), who described a technique comparing the zone diameters produced by varying concentrations of antimicrobial agents impregnated on paper discs against a test organism that could be compared on the same agar plate.

The chosen methodology is often based on many factors including the relative ease of performance, flexibility or the use of automated or semi automated devices for both identification and susceptibility testing and in some instances information regarding the clinical importance versus the interpretative results that can be provided by the method adopted (Wood and Washington, 1995).

Sensitivity tests in solid media

The gelling agent usually used for antibiotic sensitivity determination is agar to which selected nutrients are added depending on the bacterial species to be grown (Piddock, 1990). Innovation in techniques involved supplementation of medium with required compounds to the approximate physiological concentrations (Thornsberry *et al.*, 1977).

Agar diffusion is a common method adopted by many clinical laboratories in which prepared impregnated disc or a solution of antibiotic is added to a well cut in the agar (Piddock, 1990). Various factors including inoculation, medium employed, incubation conditions etc influence the diffusion zone in the antibiotic diffusion assay. The method is easy as well as better to interpret. The diameter of the zone of inhibition is plotted graphically against the logarithm of antibiotic concentration and the most appropriate disc concentration could be chosen (Piddock, 1990).

Stoke's method is used in the U. K., in which a control organism and the test organism are examined against the same antibiotic on the same plate by inoculating the control organism in the center of the plate, and test organism to the outer portion. The antibiotic discs are placed such that half of the disc lies on the control inoculum and other half on the test. The designation of sensitive or resistant is made by comparing the zone size of the test and control strains (Piddock, 1990).

Minimum inhibitory concentration (MIC)

The methods of determining MIC of an antibiotic were also developed simultaneously. Bauer *et al.* (1966) while describing the standard methods of disc susceptibility tests, compared the zone diameter with the MIC (minimum inhibitory concentration). Dilution susceptibility testing methods are used to determine the minimal concentrations usually expressed in microgram per ml of antimicrobial agents required to inhibit or kill a microorganism used. The early methods utilised dilution (usually two fold) of the antibiotics in liquid media in which the test organisms are grown (Fleming, 1929). An equivalent volume of the test organism would be added to a set of tubes that covered a concentration range of the antibiotic and after inoculation the MIC was described as the first tube in which there was no visible growth. Variations in this technique were described throughout 1940's (Fleming, 1942; Buggs *et al.*, 1946). Schmith and Reymann (1940) first described the determination of MIC of a solid media and subsequently other workers also incorporated antibiotics in to agar (Frank *et al.*, 1950).

Administration routes

The effectiveness of a drug is the function of the method of administration to fish. The important ones are oral route via medicated food, bath, dip and flush treatments, injection and topical application.

With the oral method, drugs are mixed in food and then fed to fish, which requires the addition of a calculated amount of drug in the food. The oral

method is the best way to ensure that a controlled quantity of drug is reaching the animals and has the advantage of treating mass of fish with minimal waste. But it has the following disadvantages: 1. Differential absorption rates of drug, 2. Non-palatability, 3. Non-feeding, 4. Inactivation of the drug and 5. Effect of efficacy and toxicity of certain products due to water quality.

In applications by water borne route, fishes are exposed to a solution (suspension) of the drug for a predetermined period. This could be only of few seconds' durations or to several minutes (dip) or to several hours (bath). Another method is the flush treatment in which drugs are administered in high concentrations to water in stock holding areas. In case the compounds are not soluble in water they are dispensed usually in water by surfactants (Austin *et al.*, 1981). The dosage to be given and the disposal of the spent drug are drawbacks. Injection of drug solution is useful for valuable stock, such as food fish, ornamental fish etc; but involves handling stress and is costly in time and labor (Austin and Austin, 1987). The topical application of antimicrobial compounds is worthy of consideration for valuable and pet fish and involves the same difficulties as those of injection method.

The following factors are to be considered while selecting the appropriate routes of introduction. 1.bioavailability of drug 2. Concentration at which it accumulates in target tissues 3. The rate at which it is eliminated 4. The cost of the drug 5. Potential impact on the environment and 6. Effect of water chemistry on the efficacy and toxicity of the drug (Leong, 1993).

Prospects of using antibiotics

According to Alderman and Smith (2001) the range of authorised antimicrobials, which are available for aquaculture use, is very limited and stated that with the tightening of the controls on the use of veterinary medicines and on the presence of residues in fish tissues, the range is unlikely to increase significantly. These authors also cautioned about the need to use what is available in a most efficient way to reduce the selection pressure for drug

resistance. The administration of the antimicrobial compound should be carried out after estimating the dose required for treatment, the most efficient route of administration and aspects of safety concerning the impact on health of humans and animals (Alderman and Smith, 2001).

Use of antiseptics and marine natural products in fish diseases

Marine derived bioactive compounds have begun to make significant contributions to biomedicine (Ratnasooriya *et al.*, 1990). In the recent past,many researchers carried out the observations on antimicrobial activity of marine natural products or bioactive compounds of the marine origin. The secondary metabolites of organisms such as seaweeds, sponges, gorgonids, sea cucumbers and various other invertebrates contain certain substances which are medicinally important. Observations on bioactivity of extracts of seaweeds (Rao *et al.*, 1991; Porcile *et al.*, 1991) , sponges (Kreuter *et al.*, 1992; Mary *et al.*, 1994), gorgonids (Wright, 1991) have showed the prospects of these organisms in disease management.

The usefulness of antiseptics including compounds such as acriflavin, malachite green, copper sulphate, formalin etc. in combating infections of ornamental and food fish culture are also documented by several authors (Subasinghe, 1992; Leong, 1993).

Immunisation of fish

Any disease outbreak needs immediate and considerable attention, so that the culture activity gives good results, by way of increased production. Indirectly, the health status of the cultured organism has to be ensured. One of the current criteria is to go for the specific pathogen free stock. But since this is not always possible with respect to many of the disease causing microbes including viruses, the best option is to immunise the stock against hazardous microbes, which may vary with different candidates species.

Thus immunisation became a proven and accepted tool in integrated and comprehensive programme of aquatic animal health management worldwide, since its inception over 50 years ago (Busch, 1994). The prophylactic use of vaccines significantly reduced mortality and other disease related losses, lowered the unit cost of production, reduced the use of dependence on drugs and chemicals, and improved the general overall quality of the product (Busch, 1994). An advantage of immunising fish other than to avoid the use of chemotherapeutics is to prevent the diseases, which cannot be treated with antimicrobial agents, like viral pathogens or attack by drug resistant bacteria and some protozoan parasites, and also to enhance the general health of animals by reducing chronic infections (Rohovec *et al.*, 1981). According to Plumb, (1992) vaccination of fish for disease prevention will eventually be a significant procedure in fish disease management.

Successful immunisation of fish was reported for the first time by Duff (1942) for protection of cutthroat trout with a bacterin prepared against *A. salmonicida*, whereas the commercial development of bacterins and vaccines for fish was initiated in 1973 by a cooperative research agreement between the private industry and the United States Fish and Wildlife Service (Tebbit and Goodrich, 1983). Initial research from this co-operative agreement led to the vaccine infiltration and the hyper osmotic infiltration immersion methods of administering bacterins. Most of the studies on development of bacterins were directed against Vibriosis, furunculosis, bacterial kidney disease, enteric redmouth disease and columnaris disease (Sindermann, 1990).

Preparation of bacterins

The vaccines or bacterins to be used are prepared via mainly two means- 1. Killing the antigens, 2. By attenuating the antigens. Either way the antigenic determinants of the pathogen are retained and introduced via the routes mentioned above.

In general, to prepare bacterins against vibriosis, a build up of bacterial culture is required (Rohovec *et al.*, 1981), followed by the harvest and storage in viable conditions. Killing of cells is brought about either by thermal shock in a constant water bath at 58 ^{0} C for 30 minutes or by administering formalin (wet packed whole cell bacterin) at the rate of (0.3-0.5%) or other suitable agents into the bacterial culture (Fryer *et al.*, 1978).

Methods Of Administration Of Vaccine

The common routes of administration are injection (with or without adjuvant), oral application, hyper osmotic infiltration, immersion or bath. Oral or parenteral route were thought to be the most suitable methods of administration from the beginning. Injection by hand, oral application, hyper osmotic infiltration, immersion or bath, spray or shower and automated immersion methods have been used to vaccinate fish (Tebbit and Goodrich, 1983).

Injection was first used as the safe and effective method of administration of vaccine against bacterial diseases such as vibriosis. This is particularly useful when valuable stock such as brood fish are in the picture and ensures the receipt of an exact dose of the product by the fish stocked. The prospects of this method are high with respect to its use in the highly priced ornamental fisheries. Injections can be made either intraperitoneally or intramuscularly or in the dorsal sinus.

Immersion vaccination was developed by Antipa *et al.* (1977), which has advantages over the other methods of vaccination in terms of mass vaccination, handling etc. Agglutination antibodies and celiular response were detected in fish immunised with *V. anguillarum* vaccines detected in fish immersed in *V. anguillarum* bacterins (Sakai *et al.*, 1986). Immersion is accomplished by placing fish into bacterin containing solution for varying durations.

In the hyper osmotic infiltration method, the animals first immersed in a hyper osmotic solution were subsequently immersed in solution containing antigens (two step method). This technique has gained popularity in many parts of the world and the efficacy is not limited by the size of the fish and is applied to mass immunisation (Alexander *et al.*, 1981). However it has the disadvantage of handling stress and osmotic shock, which may render sub clinical or latent infections (Amend and Johnson, 1981). The method was developed with hyper osmotic infiltration of urea and sodium chloride using a model system of infiltration of bovine serum albumin into the serum of fish (Amend and Fender, 1976).

The automated immersion, which was introduced by United States Wildlife vaccines in 1980, was another innovation in the mass administration of vaccines to fish. Fish are placed on a suitable rubber conveyer belt that transport through a reservoir of bacterin and then to a return slide. Fish of various sizes can be vaccinated by total immersion for 15 to 20 seconds at rates up to 200 pounds per minute (Tebbit and Goodrich, 1983).

To control fish diseases, oral method of vaccination was developed by Fryer *et al.* (1972). This is supposed to be the only practical method of vaccinating large numbers of fish. Concentrated antigen is incorporated into fish diet and then fed to animal for administration for several days to ensure that all the fish receive an immunising dose (Rohovec *et al.*, 1981).

Other methods of bacterin administration include the delivery of bacterins by the spray or shower method, by spraying fish with a suspension containing the bacteria. This suspension can be given with or without pressure and exposure to the solution is of shorter duration. The spray vaccination method was developed by Gould *et al.* (1978).

Evaluation of vaccines

The evaluation of efficacy or potency of the fish vaccines may be performed by reproducing the disease under controlled conditions by various challenge systems (Amend, 1981) as well as by monitoring via *in vitro* culture methods (Anderson, 1988).

According to Udey and Fryer (1978) the development of bacterins have posed challenges to many who followed up because of the following reasons:-

1. Standardisation of the challenge
2. Knowledge about the pathogen
3. Most suitable techniques (route of administration, dose) aimed at a particular disease outbreak.

However, with time, the techniques of preparation as well as administration became sophisticated and as a result, levels of protection obtained through vaccination became significant. The different kinds of vaccines presently being used are of the following types: 1. Inactivated bacterial vaccines, 2. Modified live bacterial vaccines 3. Toxoids 4. Inactivated viral vaccines 5. Modified live viral vaccines and those vaccines against rickettsia, chlamydia, hookworms and other agents (Brown, 1978).

The first vaccines to be successfully commercialised were those against *V. anguillarum*, V. *ordalii*, and *Y. ruckeri* in the late 1970s, all of them being federally licensed (Cardella and Eimers, 1990; Sindermann, 1990). Developed initially for the salmonid industry, these bacterins are now routinely used worldwide on many species of fish. Vaccines against *Vibrio salmonicida*, a pathogen of salmonids, *A. salmonicida*, a pathogen of salmonids and carp, and *E. ictaluri*, a pathogen of channel catfish have also been commercialised and are in widespread use (Newman, 1993). Adams *et al.* (1999) listed 27 commercially available fish vaccines, which could be administered by different methods. Most of these vaccines originated from the U. K., U.S.A. and Norway.

48% of the vaccines were manufactured by Norway followed by U. K. (27%) and U.S. A. (19%).

A number of other bacterins have been the subject of research and some of them may eventually be available for commercial use. Though bacterins against *V. parahaemolyticus*, *A. hydrophila* and *E. tarda*, have been successfully tested, the serologic heterogeneity of these groups of organisms remain as the constraint in the development of widely utilisable vaccines. Serologically more homogeneous groups, including *F. columnaris*, *P. piscicida*, and *Sreptococcus* spp. affecting fish, will end up in commercially available bacterins in the near future (Newman, 1993).

Vibriosis bacterins: Immunisation attempts were made to efficiently manage the potential threat posed by vibriosis (Tebbit and Goodrich, 1983). Immunoprophylaxis was found to make vibriosis amenable (Amend *et al.*, 1980). As far as *Vibrio* bacterins are concerned, it was observed by different authors that the degree of protection using different methods of administration (such as oral, injection, shower or immersion) yielded varying results (Amend and Johnson, 1981).

Spray and bath methods of administration have been found successful (Kawano *et al.*, 1984) as well as parenteral or intramuscular injections and incorporation of bacterin in food. (Tiecco *et al.*, 1988). Mass immunisation methods against vibriosis were practiced. Among these, although oral route was regarded as the easiest (Fryer *et al.*, 1972; Fryer *et al.*, 1978) protection was found to be of short duration (Amend and Johnson, 1981, Gould *et al.*, 1979) compared to other routes of administration like spray (shower) method. In the spray method, with least pressure maximum immunity was elicited (Gould *et al.*, 1978).

Methods such as vacuum and infiltration using hyper osmotic solutions were evaluated (Amend and Fender, 1976). Considering the

efficiency, the hyper osmotic infiltration method was advocated to effectively immunise fish against vibriosis (Croy and Amend, 1977). Simple immersion method also was found to provide high immunity and was noticed that immersion into vaccine containing solution is equally effective as immunisation in hyper osmotic solution and was adopted as the ideal method of delivery of vibriosis bacterins (Egidius and Anderson, 1979) avoiding the chance of osmotic shock (Harrell, 1979).

In spite of these developments, the injection method was still used in bigger fish (larger than 50 g) and many of the studies conducted indicated that larger fish always responded better to injection (Amend and Johnson, 1981). Preliminary studies taken up by National Marine Fisheries Service, on the vaccination aspects of salmonids against vibriosis, have proven that injection vaccination of killed wet packed cells are sufficient to protect Coho salmon against vibriosis (Harrell, 1978).

Aeromonas vaccines: Variable results have been obtained with studies on salmonid vaccines, both results of success and those of failures. In recent studies, attempts to develop effective killed cell vaccines (bacterins) for furunculosis have resulted in numerous commercial vaccines that induce only a partial protective immune response (Thornton, 1995). Most of the early works on immunity generated through vaccines were concentrated on salmons, with *A. salmoncida*, causative agent of furunculosis (Fryer *et al.*, 1978). Salmonid fish respond most strongly to injection administration of killed cells of *A. salmonicida* in an oil based adjuvant and there are reports of production of elevated levels of specific antibodies resulting from the injection of killed *A. salmonicida* cells (Udey and Fryer, 1978; Paterson and Fryer, 1974a; Krantz *et al.*, 1963). There are reports of trouts, which get protected from *A. salmonicida*, when immunised especially in combination with Freund's adjuvant, which was demonstrated by laboratory challenge to the extent of getting protection even when the fish were stressed (Krantz *et al.*, 1964).

Against *A. hydrophila* the efficiency of immunisation, was tried by oral, injection and immersion routes (Schachte Jr., 1978; Chen *et al.*, 1996; Newman, 1993).

Edwardsiella: Studies on immunisation of channel catfish (seven days old) with modified live *Edwardsiella* vaccine provided effective response (Shoemaker *et al.*, 1999). However, the administration of formalin-killed cells by immersion followed by oral booster did not give positive response (Thune *et al.*, 1997). In the case of catfish also, the modified live bacterins were found to be effective (Shoemaker *et al.*, 1999; Klesius and Shoemaker, 1999).

Hagerman redmouth (HRM) disease/ Enteric redmouth disease (ERM): HRM, which is an important disease of rainbow trout causing mass mortalities, was controlled by means of immunisation of the host (rainbow trout) by feeding the fish with bacterin containing diet or by bath (Ross and Klontz, 1965; Anderson and Nelson, 1974) as well as by bacterin inoculated subcutaneously (Anderson and Nelson, 1974; Krantz *et al.*, 1964). The immunisation by *Y. ruckeri* bacterins by both oral and injection routes was found to provide protection against ERM. Immersion vaccination led to protection against the disease thereby reducing mortality, which paved the way for successful control of the ERM disease by the mass immunisation programmes (Tebbit *et al.*, 1981).

Flexibacter: Columnaris disease, in pen and cage culture, was avoided by using polyvalent bacterin (Schachte, Jr., 1978), via immunisation and preliminary results showed humoral antibody responses to oral, injection and immersion routes.

Injection by intramuscular and subcutaneous, routes elicited the production of agglutination antibodies against *Chondrococcus columnaris*,

(Schachte and Mora, 1973). Successful immunisation by oral method was reported by Fugihara and Nakatani (1971).

Other pathogens: Prophylactic treatments against causative agent of pseudotuberculosis in fish have been demonstrated in yellowtail, *Seriola quiqueradiata* (Fukuda and Kusuda, 1985) by oral, spray and immersion methods of immunisation of juveniles. Effectiveness of immunisation against Streptococcus, another significant pathogen of yellowtail, has also been documented (Iida *et al.*, 1982).

Control of bacterial kidney disease caused due to *Renibacterium salmoninarum* by chemotherapy was reported as unsuccessful (Adams *et al.*, 1999) and hence experimental vaccination studies were carried out in salmonids. Paterson *et al.* (1981) and McCarthy *et al.* (1984) achieved promising results with adjuvanted and non-adjuvanted vaccines.

In addition to these developments, monovalent and multi-faction bacterins (i.e., antigenic suspensions of inactivated bacterial organisms) are available for the vaccination of fish to aid in the prevention of furunculosis caused by *A. salmonicida*, enteric septicemia of catfish caused by *E. ictaluri*, columnaris disease caused by *Flavobacterium columnare*, vibriosis caused by *V. anguillarum* and *V. ordalii*, cold water vibriosis caused by *V. salmoninarum*, and enteric red mouth diseases caused by *Y. ruckeri* (Birnbaum, 1998).

Factors affecting efficacy of vaccine

Influence of parameters such as age and size in the administration of bacterin were investigated (Amend and Johnson, 1981). Size of the fish is an important determining factor in the immunity gained through vaccination. Amend and Johnson (1981) suggested that fish must be at least 1g before they develop a high level of protective immunity. Hence, it is a function of size and not age. It is also demonstrated that, the level and duration of immunity increases in fish that were vaccinated at larger size (Tebbit and Goodrich, 1983). In addition, the

total number, value of fish to be immunised, along with the environmental parameters were reported to limit the efficacy. Water temperature has been found to influence the development of protective immunity (Bisset, 1948; Paterson and Fryer, 1974 b).

Future Needs/Prospects

The important considerations derived out of the retrospective research on immunisation studies are:

1. The techniques of inactivation of the pathogen (killed vaccines)
2. Degree of attenuation (modified live vaccines)
3. Standardisation of the optimum dose in terms of density of cells. This will help to obtain a balance between safety and effectiveness,
4. Aspects of shelf life and lyophilisation of the product for long term storage
5. The best routes and techniques of administration
6. The dangers of hypersensitivity
7. Other problems dealing with marketing aspects.

Geographical location is an important factor, since most of the pathogens encountered in aquaculture are endemic or known to occur in a particular geographic area. Effective vaccines against a number of potential diseases, which are endemic, have been developed and successful vaccination programmes have been taken up in Norway, Greece, Turkey and other Mediterranean regions (Anon, 1998). In addition, disease surveillance programmes will be required to determine the disease profiles of aquaculture facilities.

Identification of factors such as role of stress, other infectious processes and various environmental factors, along with suitable measures of development of the bacterial vaccines integrating all the related factors will help to develop better immunisation strategies against dreaded pathogens.

According to Newman (1993), the use of a new generation of adjuvants in conjunction with automated injection methods could result in vaccines that will protect against diseases for which conventional methods may not be successful, such as bacterial kidney disease (BKD) caused by *Renibacterium salmoninarum*. Moreover, serotyping of fish pathogens, development of artificial challenge systems and better delivery systems especially when large populations are dealt with, needs consideration for successful immunisation. Some of the recent developments in fish vaccination include 1. The genetic recombination 2. Genetic attenuation 3. Protein engineering and 4. anti-idiotypic vaccines (Ellis, 1988). A significant advantage of genetically engineered vaccines is the ability to construct multivalent preparations, which protect against several pathogens or different strains of the same pathogen (Winton, 1998).

The storage aspects and marketing of the product,especially in the case of multifraction products needs much emphasis (Tebbit and Goodrich, 1983). This will help to evolve efficient bacterins as well as to administer the bacterin at the most appropriate time. Although a few vaccines are commercially available at present, considering the awareness and the advantages of immunisation, there may be high demand for the bacterins in future (Rohovec *et al.*, 1981).

With this background knowledge on the control of marine fish diseases, in the present chapter, both *in vitro* and *in vivo* studies were conducted to evaluate:

1. The antibiotic sensitivity of selected isolates
2. *In vitro* inhibitory activity of chosen antiseptic compounds against the selected isolate
3. *In vitro* inhibitory activity of certain marine natural products against the selected isolate.
4. Immunoprophylactic studies against the selected pathogenic isolate using bacterins (formalin killed cells).

Experiments on the Susceptibility of Bacterial Isolates to Antibiotics

Isolates obtained from the moribund fish and recently dead ornamental fish were used for screening the antibiotics. 31 selected isolates were used for studying the sensitivity pattern. The list of the isolates used for the studies and their sources are given in Table 11.

The antibiotic discs used and their concentrations are given in Table 12. They belonged to penicillins, aminoglycosides, streptomycin, macrolides, quinilones, sulphonamides potentiated sulphonamides, nitrofurans, tetracyclines and unclassified groups.

Bauer's (1966) disc diffusion method was used of for finding the sensitivity pattern of the antibiotics against the selected isolates. The antibiotic agar medium was pour plated and allowed to set. After cooling, 0.1 ml of inoculum from a fresh peptone broth culture was spread on the agar plate. Five to six antibiotic discs (HiMedia) were placed on the plate each one about 2.5 cms apart.

The plates were incubated for 24 hours at 37^{0}C and after the incubation period the diameter of the sensitivity zones were measured and recorded as resistant (R), sensitive (S) and intermediate (I). The percentage sensitivities towards various compounds were calculated. The antibiogram of all the isolates studied was also prepared.

Experiments on Minimum Inhibitory Concentration (MIC)

The antibiotic compounds for MIC studies were selected based on their activity in the disc diffusion assay. Those compounds, which exhibited high inhibitory activity towards the selected pathogenic isolate, *S. marcescens*

Table 11. Isolates used for antibiotic sensitivity studies

Isolate code	Species	Source
9-98	*Alcaligenes*	*Acanthurus*
20-98V	*V. furnissi*	*Acanthurus*
29-98	*Pseudomonas*	*Acanthurus*
72-98	*Flavobacterium*	Damsel
103-98	*Alcaligenes*	*Amphiprion*
103-98A	Enterobacteriaceae	*Amphiprion*
103-98B	*Acinetobacter*	*Amphiprion*
115-98	*Pseudomonas*	Damsel
1-99	*Acinetobacter*	*Acanthurus*
4-99	*Acinetobacter*	*Apogon*
41-99	*Pseudomonas*	Serranid
50-99A	*Flavobacterium*	*Heniochus acuminatus*
50-99	*V. mediterranei*	*Heniochus acuminatus*
61-99	*Pseudomonas*	*Amphiprion*
61-99A	*V. mediterranei*	*Amphiprion*
61-99B	*V. mediterranei*	*Amphiprion*
68-99	Enterobacteriaceae	*Chaetodon*
20-98R	*S. marcescens*	*Acanthurus*
68-99C	*Flavobacterium*	*Chaetodon*
73-99	*V. mediterranei*	*Chaetodon*
117-99	*Flavobacterium*	Squirrel fish
124-99	*V. furnissi*	*Acanthurus*
151-99A	*Acinetobacter*	*Chaetodon*
151-99B	*S. marcescens*	*Chaetodon*
151-99C	*Alcaligenes*	*Chaetodon*
CFY	*Flavobacterium*	*Amphiprion*
CY	*Flavobacterium*	*Amphiprion*
LJ	*Flavobacterium*	*Apogon*
OE	*Alcaligenes*	*Apogon*
8-2K	*Flavobacterium*	*Amphiprion*
9-2K	*Flavobacterium*	*Amphiprion*

Table 12. List of antibiotic compounds used

Antibiotic disc	Concentration (µg/disc)
Penicillin	2.5 I.U./disc
Ampicillin (A^{25})	25
Cloxacillin (Cx^{10})	10
Neomycin (N^{30})	30
Kanamycin (K^{30})	30
Gentamycin (G^{10})	10
Erythromycin (E^{15})	15
Streptomycin (S^{10})	10
Chlorampenicol (C^{25})	25
Rifampicin (R^{15})	15
Nalidixic acid (Na^{30})	30
Amphotericin B (Ap^{100})	100
Bacitracin (B^{10})	10 units/disc
Doxycycline (Do^{30})	30
Co-trimoxazole (Co^{25})	25
Metronidazole (Mt^{5})	5
Sulphamethizole (Sm^{300})	300
Sulphadiazine (Sz^{100})	100
Trimethoprim (Tr^{5})	5
Ciprofloxacin (Cf^{10})	10
Furazolidone ((Fr^{50}))	50
Nitrofurazone (Nr^{100})	100
Nitrofurantoin (Nf^{300})	300
Tetracycline (T^{30})	30
Oxytetracycline (O^{30})	30

indicated by zone diameter, were selected. For MIC studies, the tube dilution method (Finegold and Martin, 1982) was followed. Antibiotic compounds were dissolved in the recommended diluents and final drug concentrations ranging from 0.5 µg to 100 µg were prepared in the final diluents with the addition of a uniform amount of the broth (inoculum). The prepared solutions in test tubes were kept for incubation at 37 ^{0}C and after incubation period, the test tubes were examined for turbidity. Lowest concentration of the drug that produced no visible growth was taken as the MIC.

Determination of Minimum Bactericidal Concentration (MBC)

For estimating minimum bactericidal concentration (MBC), 0.1ml inoculum from the tubes, which did not exhibit turbidity were spread plated on agar plates and incubated. Lowest concentration of the drug out of the tubes without turbidity or visible growth, which on plating gives no growth, was taken as the MBC.

Experiments on Susceptibility of Bacterial Isolates to Antiseptic Compounds

The antiseptic compounds used for the *in vitro* activity studies are given in Table 13. Each compound was weighed and three different concentrations (10, 100µg and 1000µg) were made use of for the *in vitro* activity. Inoculum from a 24-hour broth culture of the selected pathogenic isolate, *S. marcescens* was swabbed on the agar surface and wells were cut in the medium using a cork borer of 6mm diameter. Wells were sealed at the bottom with 1% agar, which was allowed to cool. The compounds were added to wells as per three different concentrations mentioned above. After incubation at 37 ^{0} C the zones of inhibition around the wells containing the compounds were measured and recorded.

Table 13. List of antiseptic compounds used in the study

Antiseptic compounds	Concentration		
Potassium permanganate	10µg	100µg	1000µg
Malachite green	10µg	100µg	1000µg
Methylene blue	10µg	100µg	1000µg
Sodium chloride	10µg	100µg	1000µg
Formalin	10µl	100µl	1000µl
*Virkon	10µg	100µg	1000µg
Copper sulphate	10µg	100µg	1000µg
Acriflavin	10µg	100µg	1000µg
Benzalkonium chloride	10µg	100µg	1000µg

*Virkon- Conmposition:

Sodium chloride	1.5% w/w
Salts containing potassium monophosphate, Potassium hydrogen sulphate	49.8% w/w.

Experiments on Susceptibility of Bacterial Isolates towards Chosen Marine Natural Products

Eight different extracts of sponges and seaweeds (source: ICAR Ad-hoc scheme, Vizhinjam R.C. of CMFRI) were used for the *in vitro* inhibitory studies against the selected isolate, *S. marcescens*. The marine natural products used for *in vitro* activity studies are given in Table 14.

The extracts were tested for their *in vitro* inhibitory activity by the zone diffusion method. Wells were cut in nutrient agar plate swabbed with a fresh culture of the selected isolate and were sealed at the bottom with 1% agar. The extracts were added to the wells and incubated at 37^{0}C. Methanol was added to the control wells. Following incubation the zones of inhibition around the wells were measured and recorded.

In vivo Immunoprophylactic studies using bacterins (Autogenous Vaccines)

Experimental set up

Clown fish (*A. sebae*) was used as the experimental fish. The study was carried out in the same brood of 3 months age fish stocked in 5 tonne tanks. The prepared bacterin was administered to the whole stock consisting of 50 fish at the rate of 10^5 cells per gram, which was the lethal dose estimated from pathogenicity studies. The fish were monitored continuously for mortality and response pattern.

Preparation of bacterin

The bacterial pathogenic isolate (*S. marcescens*) selected for pathogenicity studies was used for bacterin preparation. Bacterin (autogenous vaccine) was prepared from the isolate as per the following scheme.

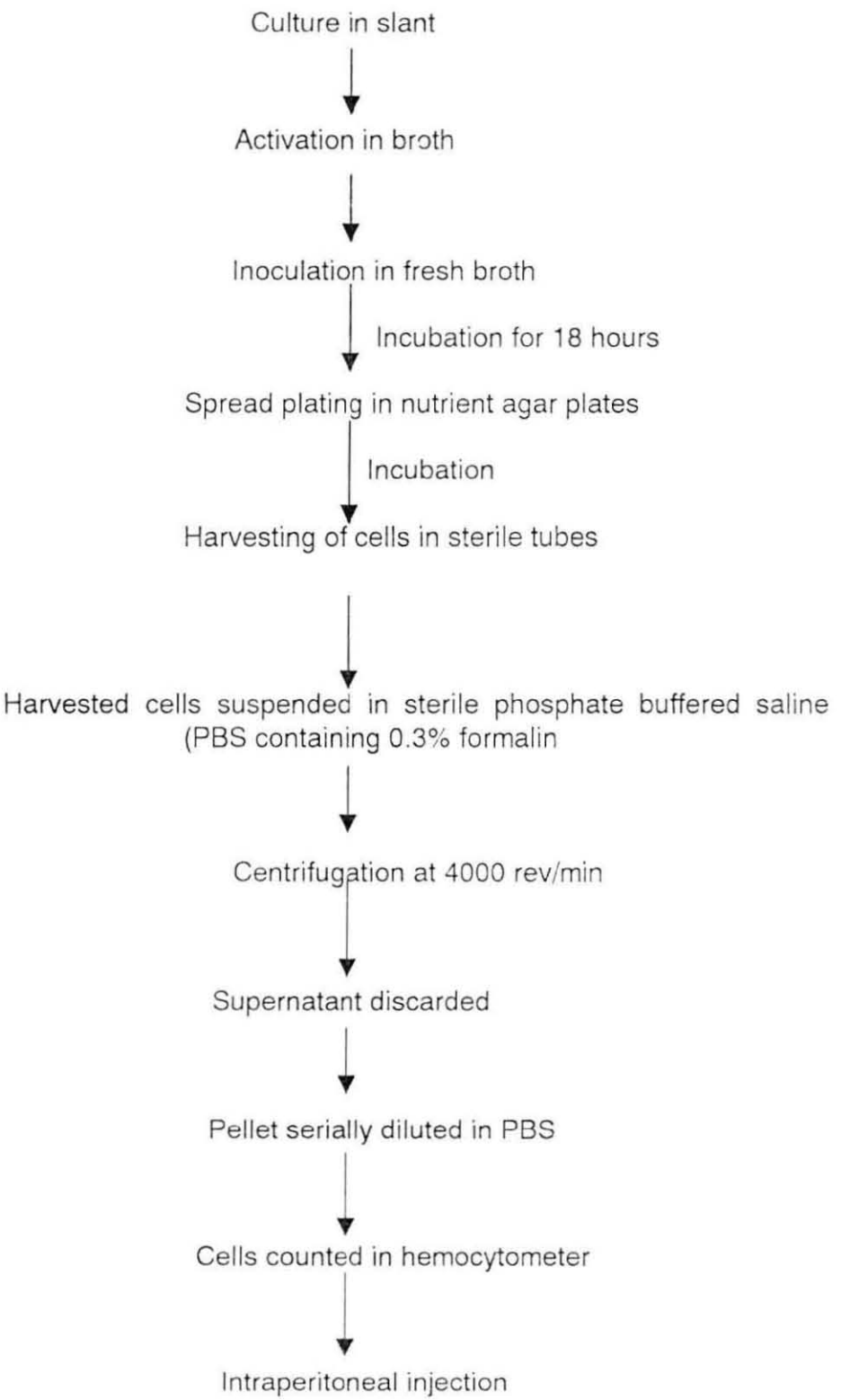

Culture in slant
Activation in broth
Inoculation in fresh broth
Incubation for 18 hours
Spread plating in nutrient agar plates
Incubation
Harvesting of cells in sterile tubes
Harvested cells suspended in sterile phosphate buffered saline (PBS containing 0.3% formalin
Centrifugation at 4000 rev/min
Supernatant discarded
Pellet serially diluted in PBS
Cells counted in hemocytometer
Intraperitoneal injection

Table 14. List of marine natural products used and the solvents used for extracts

Extract source	Solvent
Hypnea musciformis	Methanol
Valanopsis pachyderma	Methanol
Chnoospora maxima	Methanol
Caulerpa scalpelliformis	Methanol
Gracilaria corticata	Methanol
Gracilaria fergusoni	Methanol
Sigmodocea carnosa	Methanol
Callyspongia	Methanol
PVR- sponge extract	Methanol

Challenge studies

For challenge studies, the fish were transferred to experimental FRP tanks of 1 tonne capacity. After 15, 30 and 50 days of administration of bacterin, experimental fish were subjected to challenge studies. The injected fish were given intraperitoneal injection of live pathogenic cells at the lethal dose which was earlier determined as 10^5 cells per gram, from pathogenicity studies. A set of control fish was given injection of 0.85% saline. After injection, fish were monitored for mortality as well as clinical signs and behavioral responses.

The percentage relative protection (PRP) rendered by the administration of bacterin was calculated by the following formula (Amend, 1981).

$$PRP = 1 - \frac{\text{Percentage of dead (immunised)}}{\text{Percentage of dead (non immunised)}} \times 100$$

Susceptibility of bacterial isolates to antibiotic compounds

The percentage sensitivity pattern of the 25 antibiotic compounds tested is given in Table 15. The detailed results of the zone of inhibition as well as the status of sensitivity are given in Appendix 2. Out of the 25 antibiotics tested, maximum number of the isolates were found to be sensitive to aminoglycosides like gentamycin and a wide variety of sulphonamides and miscellaneous groups of antibiotics.

The sensitivity pattern in the descending order is gentamycin > streptomycin and tetracycline> nalidixic acid > ciprofloxacin > oxytetracycline> kanamycin, rifampicin, doxycycline, co-trimoxazole > chloramphenicol > sulphamethoxazole, trimethoprim, nitrofurantoin and cloxacillin> ampicillin > nitrofurazone and furazolidone > neomycin, erythromycin > sulphadiazine > penicillin > amphotericin B and bacitracin > metronidazole. The sensitivity pattern of various isolates towards the compounds tested is as given below.

Penicillin (2.5 I.U./disc): The sensitivity towards penicillin served as an important criterion for identification of bacterial pathogens. Most of the isolates were found resistant to this compound (74.19%).

Ampicillin (25 µg/disc): Isolates of *V. furnissi* and *Acinetobacter* showed 100% sensitivity towards ampicillin (25µg/disc) whereas 75% of the isolates of *V. mediterranei*, *Alcaligenes* and *Pseudomonas* showed sensitivity.

Cloxacillin (10µg/disc): *Acinetobacter*, *V. furnissii*, *V. mediterranei* and *Pseudomonas* showed 100% sensitivity to cloxacillin (10µg/disc).

Table 15. Antibiotic sensitivity pattern of the tested isolates

Antibiotic used	Percentage of sensitive isolates	Percentage of isolates showing intermediate sensitivity	Percentage of resistant isolates
Penicillin (2.5 I.U/disc)	16.13	9.68	74.20
Ampicillin (A^{25})	58.1	3.23	38.71
Cloxacillin (Cx^{10})	64.52	12.90	22.58
Neomycin (N^{30})	51.61	32.26	16.13
Kanamycin (K^{30})	80.65	3.23	16.13
Gentamycin (G^{10})	96.77	0	3.24
Erythromycin (E^{15})	51.61	29.03	19.35
Streptomycin (S^{10})	93.55	0	6.45
Chlorampenicol (C^{25})	74.20	6.45	19.35
Rifampicin (R^{15})	80.65	12.90	6.45
Nalidixic acid (Na^{30})	90.32	0	9.68
AmphotericinB (Ap^{100})	12.90	0	90.32
Bacitracin (B^{10})	12.90	16.13	70.97
Doxycycline (Do^{30})	80.65	6.45	12.90
Co-trimoxazole (Co^{25})	80.65	0	19.35
Metronidazole (Mt^{5})	0	6.45	93.55
Sulphamethizole (Sm^{300})	64.52	0	35.48
Sulphadiazine (Sz^{100})	45.16	9.68	45.16
Trimethoprim (Trt^{5})	64.52	0	35.48
Ciprofloxacin (Cf^{10})	87.10	0	12.90
Furazolidone ((Fr^{50})	54.84	9.68	35.48
Nitrofurazone (Nr^{100})	54.84	0	45.16
Nitrofurantoin (Nf^{300})	64.52	22.58	12.90
Tetracycline (T^{30})	93.55	3.23	3.23
Oxytetracycline (O^{30})	83.87	9.68	6.45

Neomycin (30 µg/disc): 50% of the isolates of all genera showed sensitivity to neomycin (30 µg/disc). *Acinetobacter*, *V. furnissi*, *V. mediterranei*, *Pseudomonas* and members of enterobacteriace showed 100% sensitivity to this compound.

Kanamycin (30 µg/disc): A minimum of 50% sensitivity was shown towards kanamycin (30 µg/disc). *Acinetobacter*, *V. mediterranei*, *V. furnissii*, members of enterobacteriaceae and *S. marcescens* were found to show 100% sensitivity to kanamycin.

Gentamicin (10 µg/disc): All the genera except *Flavobacterium* showed 100% sensitivity to Gentamicin whereas *Flavobacterium* showed 88.8% sensitivity.

Erythromycin (15µg/disc): 50% of the isolates of all genera were sensitive to erythromycin. In the case of *Acinetobacter*, *V. mediterranei*, *Pseudomonas* and *S. marcescens* all the isolates were sensitive.

Streptomycin (10 µg/disc): In the case of streptomycin, more than 50% of isolates of all the genera showed sensitivity. Isolates of *Alcaligenes*, *Acinetobacter*, *V. furnissii*, *Pseudomonas*, *Serratia marcescens* and enterobacteriaceae family showed 100% sensitivity.

Cholamphenicol (25 µg/disc): More than 75% of all the isolates of all genera showed sensitivity towards chloramphenicol. Only *Alcaligenes*, *S. marcescens* and members of Enterobacteriaceae had100% sensitivity.

Rifampicin (15 µg/disc): More than 50% of all the isolates of all genera were sensitive to rifampicin while 100% sensitivity was expressed by all the isolates except those of *V. furnissi* and *S. marcescens*.

Nalidixic acid (30 µg/disc): Members of Enterobacteriaceae were found to be resistant to nalidixic acid where all the isolates of other genera except those of *Alcaligenes* showed 100% sensitivity.

Amphotericin B (100 µg/disc): Sensitivity was exhibited by less than 50% of the tested isolates of all genera. All the isolates of *Acinetobacter*, *Flavobacterium*, *V. furnissi* and *S. marcescens* were found to be resistant to amphotericin B.

Bacitracin (10 units/disc): Less than 50% of all the isolates of all genera showed sensitivity to this compound. All the isolates of *Alcaligenes*, *V. furnissi* and *S. marcescens* isolates showed resistance to this compound.

Doxycycline (10 µg/disc): The isolates showed variable sensitivity towards doxycycline. More than 50% of isolates of all the genera except *Acinetobacter* showed sensitivity to this compound.

Co-trimoxazole (25 µg/disc): More than 75% of isolates of all genera showed sensitivity to co-trimoxazole.

Metronidazole (5 µg/disc): Except the *Acinetobacter* spp. (25%) and members of Enterobacteriaceae (50%), all other isolates were resistant to metronidazole.

Sulphamethizole (300 µg/disc): 50- 75% of all the isolates of all genera were found sensitive to sulphamethizole.

Sulphadiazine (100 µg/disc): All the isolates of *Flavobacterium* showed sensitivity to sulpahdiazine whereas 22.2 to 75% of isolates of other genera showed sensitivity to this compound.

Trimethoprim (5 µg/disc): More than 50% of the isolates of all genera showed sensitivity to trimethoprim. All the isolates of *S. marcescens* and *V. furnissi* were sensitive.

Ciprofloxacin (10 µg/disc): All the isolates of *Acinetobacter*, *V. mediterranei*, *Pseudomonas*, *S. marcescens* and members of enterobacteriaceae were found to be sensitive to ciprofloxacin. 50% of *V. furnissi* isolates, 75% of *Alcaligenes* isolates and 77.7% of *Flavobacterium* isolates were sensitive.

Furazolidone (50µg/disc): 50 to 75% of the tested isolates of all genera were sensitive to furazolidone.

Nitrofurazone (100 µg/disc): 100% of the isolates of *V. mediterranei* were sensitive while all the isolates of *Alcaligenes* were resistant to nitrofurazone. 25 to 75% of the isolates of other genera showed sensitivity.

Nitrofurantoin (300 µg/disc): All the isolates of *Acinetobacter*, *V. mediterranei*, *V. furnissii*, *Pseudomonas*, *Serratia marcescens* and Enterobacteriaceae family showed sensitivity to nitrofurantoin.

Tetrcycline (30 µg/disc): All the isolates excepting those of *V. furnissi* showed sensitivity towards tetracycline, whereas 50% of the isolates of *V. furnissi* were sensitive to tetracycline.

Oxytetracycline (30 µg/disc): All the isolates except those of *Alcaligenes* showed sensitivity to OTC;50% *Alcaligenes* isolates were resistant to OTC.

The antibiogram of the tested antibiotics is given in Table 16. The antibiotics sensitivity pattern of the pathogenic isolate, *S. marcescens* is given in Table 17. The response of this pathogen to many of the antibiotics were negative, and was sensitive to compounds including ciprofloxacin, trimethoprim, co-trimoxazole, gentamycin, tetracycline, nalidixic acid, erythromycin, choloramphenicol, streptomycin, nitrofurantoin followed by erythromycin, furazolidone, sulphadiazine and kanamycin with intermediate sensitivity (plates 11 &12).

Results of MIC and MBC evaluation

Results of MIC and MBC evaluation for the seven selected antibiotics are given in Table 18. From the studies, it was observed that the MIC value of furazolidone and streptomycin were found to be high, both requiring a concentration of 120 µg/ml for total inhibition of the isolate. All the other compounds which were selected for study had their MIC's ranging from 4 to 20 µg/ml. MBC values ranged from 10 to 200 µg/ml.

Table 16. Antibiogram of the important genera of bacterial isolates

	Concentration per disc	*Alcaligenes*	*Acinetobacter*	*Flavobacterium*	*V.furnissi*	*V. mediterranei*	*Pseudomonas*	Enterobacteriaceae	*S. marcescens*
Penicillin	2.5i.u.	75	0	44.4	0	0	25	0	0
Ampicillin	25µg	25	100	44.4	100	75	75	50	50
Cloxacillin	10µg	50	100	66.6	100	100	100	50	50
Neomycin	30µg	75	100	66.6	100	100	100	100	50
Kanamycin	30µg	50	100	77.7	100	100	75	100	100
Gentamycin	10µg	100	100	88.8	100	100	100	100	100
Erythromycin	15µg	50	100	77.7	50	100	100	50	100
Streptomycin	10µg	100	100	88.8	100	75	100	100	100
Chloramphenicol	25µg	100	75	77.7	50	75	75	100	100
Rfampicin	15µg	100	100	100	50	100	100	100	50
Nalidixic acid	30µg	75	100	100	100	100	100	0	100
Amphotericin B	100µg	25	0	0	0	25	25	50	0
Bacitracin	10 u	0	25	33.3	0	50	50	50	0
Doxycycline	30µg	100	25	88.8	100	100	100	50	50
Co-trimoxazole	25µg	75	100	88.8	50	75	75	50	100
Metronidazole	5µg	0	25	0	0	0	0	50	0
Sulphamethizole	300µg	50	75	77.7	50	50	75	50	50
Sulphadiazine	100µg	75	50	22.2	100	75	75	0	50
Trimethoprim	5µg	50	50	77.7	100	50	50	50	100
Ciprofloxacin	10µg	75	100	77.7	50	100	100	100	100
Furazolidone	50µg	50	50	77.7	50	75	50	50	100
Nitrofurazone	100µg	0	25	77.7	50	100	50	50	50
Nitrofurantoin	300µg	75	100	66.6	100	100	100	100	100
Tetracycline	30µg	100	100	100	50	100	100	100	100
Oxytetracycline	30µg	50	100	100	100	100	100	100	100

Table 17. Antibiotic sensitivity pattern of *S. marcescens*

Antibiotic used	Response of *S. marcescens*
Penicillin (2.5 I.U/disc)	R
Ampicillin (A^{25})	R
Cloxacillin (Cx^{10})	R
Neomycin (N^{30})	R (16)
Kanamycin (K^{30})	I (16)
Gentamycin (G^{10})	S (23.5)
Erythromycin (E^{15})	I (20)
Streptomycin (S^{10})	S (17)
Chlorampenicol (C^{25})	S (20)
Rifampicin (R^{15})	R
Nalidixic acid (Na^{30})	S (22)
AmphotericinB (Ap^{100})	R
Bacitracin (B^{10})	R
Doxycycline (Do^{30})	R
Co-trimoxazole (Co^{25})	S (24)
Metronidazole (Mt^{5})	R
Sulphamethizole (Sm^{300})	R
Sulphadiazine (Sz^{100})	I (16)
Trimethoprim (Tr^{5})	S (24)
Ciprofloxacin (Cf^{10})	S (32)
Furazolidone ((Fr^{50})	1(18)
Nitrofurazone (Nr^{100})	R
Nitrofurantoin (Nf^{300})	S (17)
Tetracycline (T^{30})	S (23)
Oxytetracycline (O^{30})	S (24)

R - Resistant

S - Sensitive

I - Intermediate

Figures in parentheses indicate the diameter of the zone of inhibition.

Plate 11. Results of Antibiotic sensitivity of *S. marcescens*

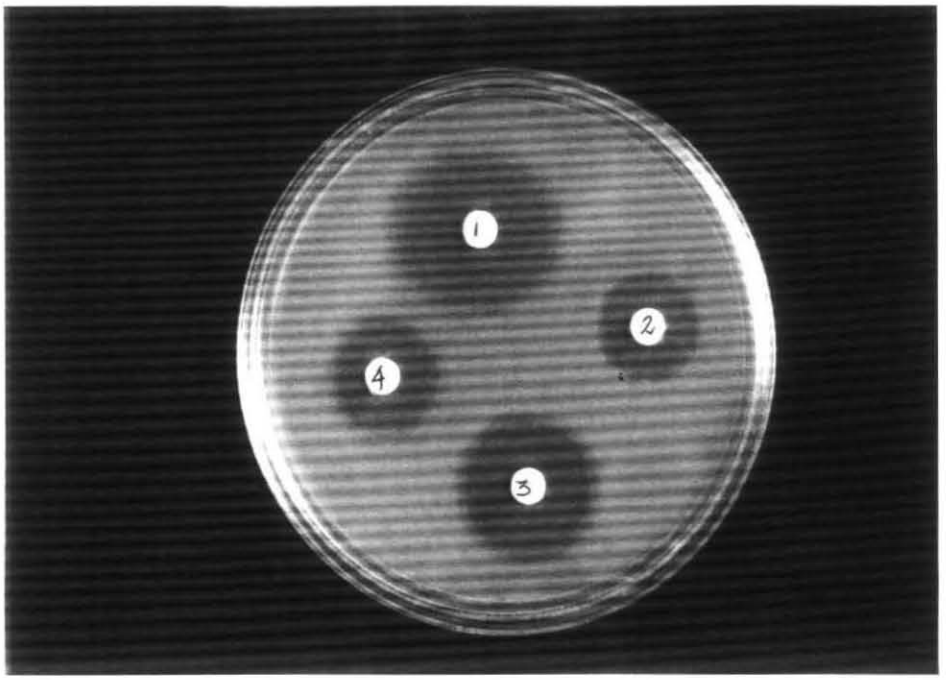
(Plates showing sensitivity)

1. Ciprofloxacin (Cf^{10})
2. Gentamycin (G^{10})
3. Oxytetracycline (O^{30})
4. Nalidixic acid (Na^{30})

Plate 12. Results of Antibiotic sensitivity of *S. marcescens*

(Plates showing intermediate sensitivity and resistance)

1. Sulphadiazine (Sz^{100})
2. Furazolidone ((Fr^{50})
3. Sulphamethizole (Sm^{300})


1
2
3
4

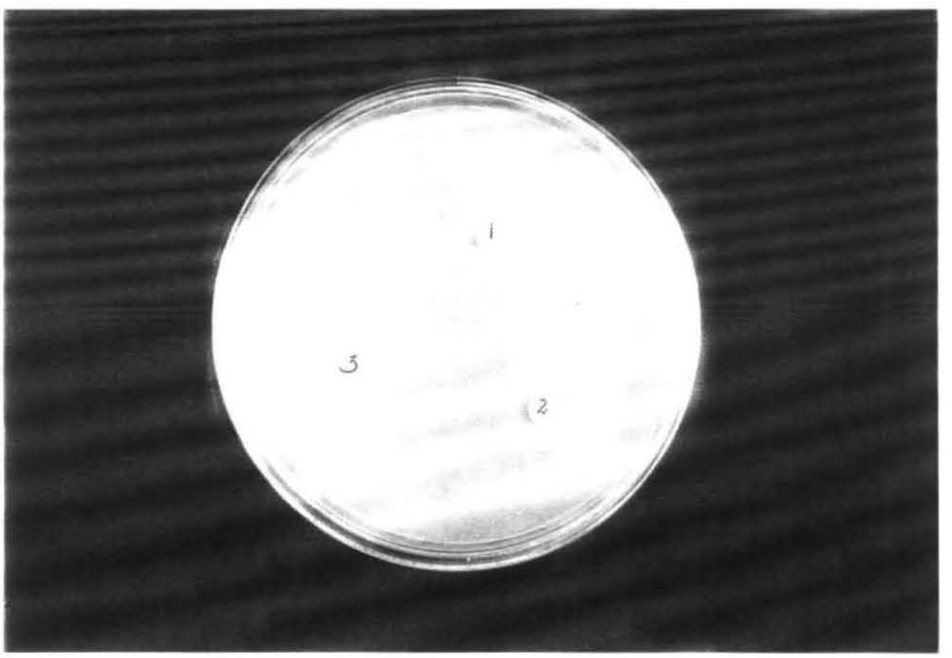

1
2
3

Table 18. MIC and MBC values of chosen antibiotics to *S. marcescens*

Antibiotic compound used	MIC (μg/ml)	MBC (μg/ml)	Sensitivity of Experimental isolate, *S. marcescens*
			Zone diameter (mm)
Tetracycline	16	36	23
Gentamycin	4	24	23.5
Furazolidone	120	200	18
Oxytetracycline	20	40	24
Streptomycin	120	200	17
Trimethoprim	6.66	20	24
Nalidixic Acid	8	10	22
Ciprofloxacin	4	10	32

R = Resistant

Susceptibility towards Antiseptic Compounds

The results for inhibitory activity of antiseptic compounds against the selected isolate *S. marcescens* are given in Table 19. It can be noted that formalin was highly inhibitory even at low concentrations of 10 μg/ ml in which it completely inhibited the growth of the isolate. Other compounds, which exhibited antagonistic activity were malachite green and acriflavin. No other compounds were effective in inhibiting the growth in the *in vitro* experiments.

Susceptibility towards Marine Natural Products

The marine natural products (extracts) used and their activities against the selected isolate are given in Table 20.

The inhibitory activity of *Gracilaria corticata* (a green seaweed) and sponge extracts (*Callyspongia* and *Sigmodocea carnosa*) were found to be high as indicated by the diameters of the clear zone of inhibition of growth of the pathogen. (Plates 13, 14).

Results of *in vivo* Immunoprophylactic Experiments

Results of the experiments using bacterin conducted *in vivo* indicated that the administration of formalin-killed cells (bacterin) was capable of providing immunity even on 50th day of administration (Fig. 19) without any booster dose in between. The results are summarised in Table 21.

It was found that the percentage relative protection (PRP) rendered by the administration of bacterin to the experimental fish went on increasing after the 15th day and 100% protection was achieved from challenge at 35th and 50th days. The non-immunised fish showed a morality percentage of 80 at 15th day and 50% each on 35th and 50th day of administration.

Table 19. Susceptibility pattern of *S. marcescens* towards antiseptic compounds

Compound	Sensitivity		
	1000 (µg)	100 (µg)	10 (µg)
Potassium permanganate	-	-	-
Malachite green	+ (33mm)	+ (17mm)	(12mm)
Methylene blue	-	-	-
Sodium chloride	-	-	-
Formalin	++ (No growth)	++ (No growth)	++ (No growth)
Virkon	-	-	-
Copper sulphate	-	-	-
Acriflavin	+ (13mm)	-	-

+ - active

++ - highly active (complete inhibition)

Table 20. Susceptibility pattern of *S. marcescens* towards marine natural products

Extract source*	Sensitivity	Zone diameter (mm)
Hypnea musciformis	+	14
Valanopsis pachyderma	+	24
Chnoospora maxima	+	10
Caulerpa scalpelliformis	-	-
Gracilaria corticata	+	36
Gracilaria fergusoni	-	-
Sigmodocea carnosa	+	22
Callyspongia	+	25
PVR- sponge extract	-	25

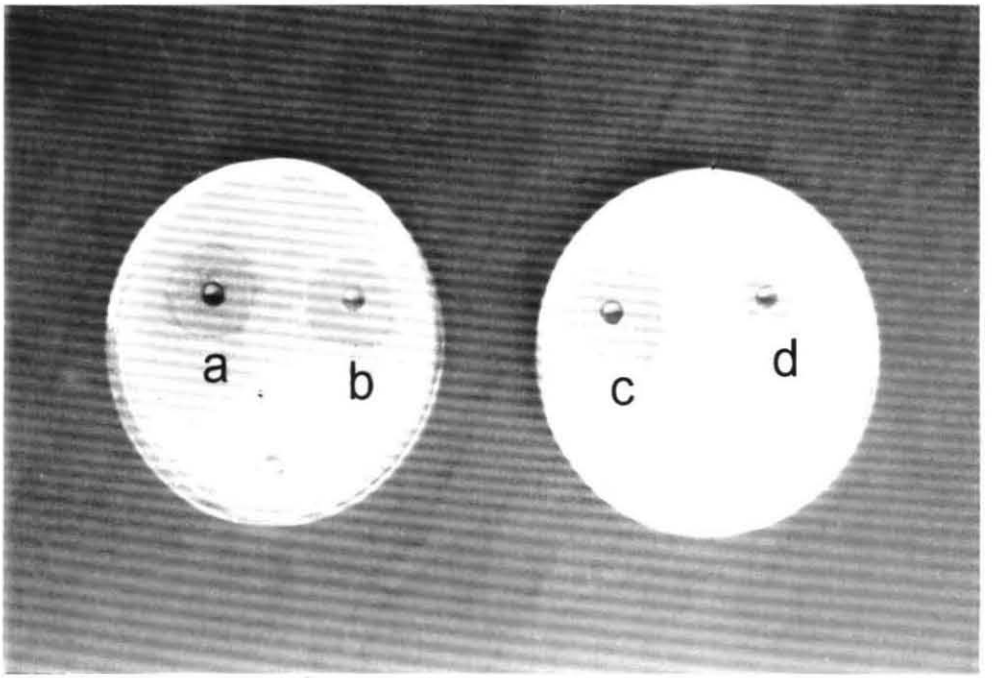
+ - sensitive

- - resistant

* Source: I.C.A.R. Ad-hoc project, Vizhinjam.

Plate 13. Inhibition of *Serratia* by bioactive compounds
(a - *Hypnea musciformis*, b- *Valanopsis pachyderma*,
c - PVR sponge extract, d - *Callyspongia*)

Plate 14. Inhibition of *S. marcescens* by bioactive compounds
(e - Sigmodocea carnosa, f - Gracilaria corticata)


a
b
c
d

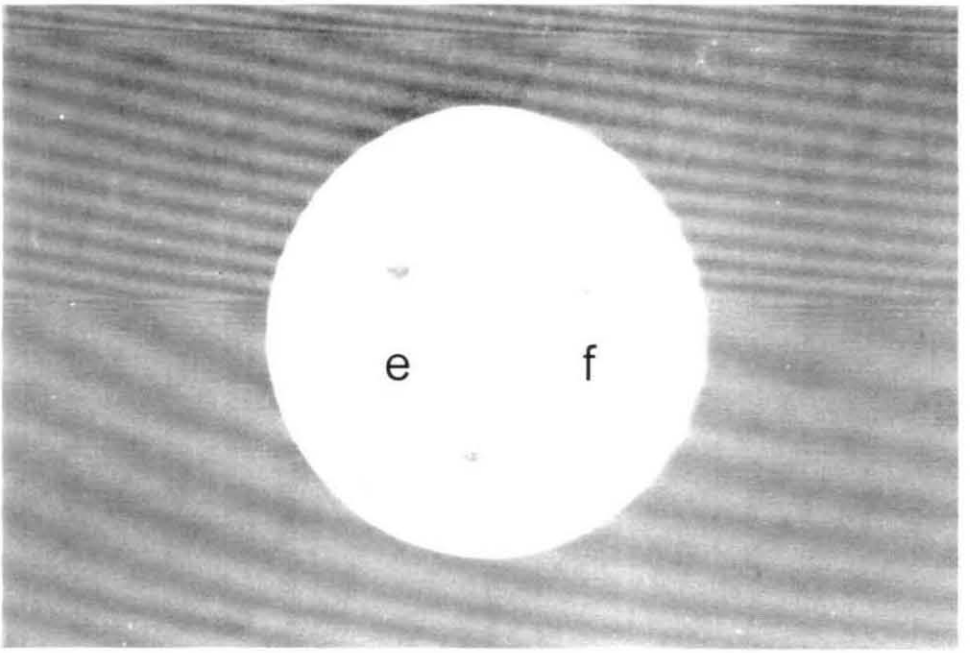

e
f

Table 21. Results of survival of clown fish on challenge with *S. marcescens* (Percentage survival and PRP)

	15th day		35th day		50th day	
	Percentage Survival	PRP	Percentage Survival	PRP	Percentage Survival	PRP
Immunised	80	75%	100	100%	100	100%
Non immunised (Control)	20		50		50	

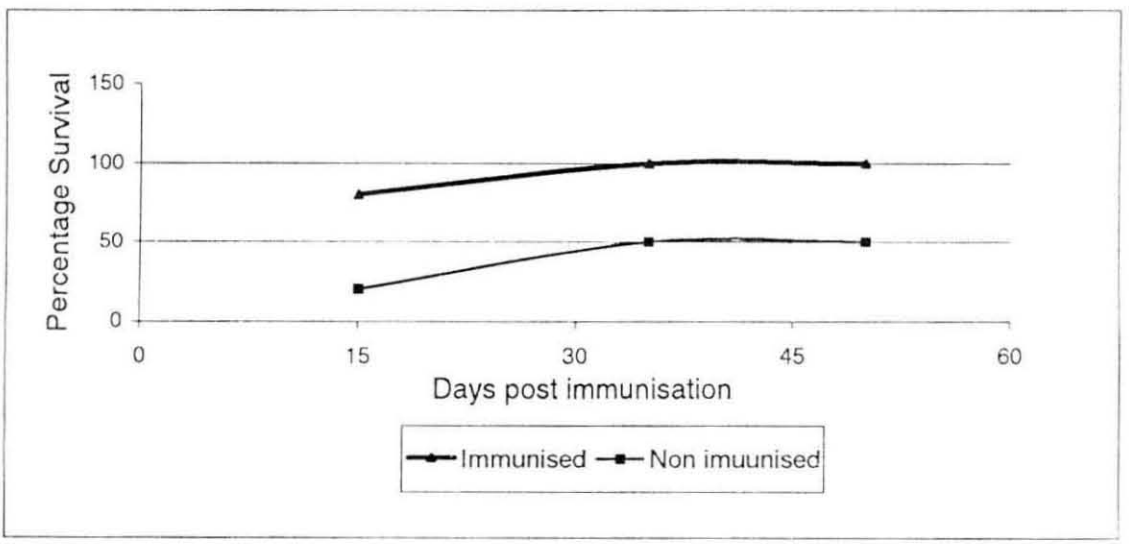


Fig. 19. Percentage survival and PRP of immunised and non-immunised clown fish

DISCUSSION

The control of bacterial infections in marine aquaria is best effected with antibiotics specific for Gram negative bacilli combined with good quality hygiene. In addition to antibiotics, use of immunostimulants in the form of bacterins as well as application of marine bioactive compounds from seaweeds and sponges (autogenous vaccines and synthetic and natural antibacterial compounds) were evaluated.

Sensitivity to antibiotics

Chemotherapy is reported as an important element of disease control among marine fish, which is useful when other measures fail. But chemotherapy demands precision of application, and is subject to decreasing effectiveness because of drug resistance (Sindermann, 1990). In the case of marine ornamental fish rearing, which is at present an upcoming enterprise, management of disease using such compounds was less documented. Hence comparative accounts on antibiotic sensitivity pattern of the isolates are not available. The influence of antimicrobial agents, particularly antibiotics in reducing the bacterial numbers in marine rearing facilities has been given by several authors (Gilmour *et al.*, 1975; Novotny, 1978; Austin *et al.*, 1981). Most of the recommended medications available for bacterial diseases are antiseptic compounds including acriflavin (for disinfection), or monacrin (mono amino acridine) in the case of early outbreak of bacterial diseases due to *Pasteurella*, *Vibrio*, *Aeromonas* and other Gram negative pathogens whereas antibiotics are not recommended due to general ineffectiveness in salt water, danger of emergence of resistant strains and the effects on filters (Emmens, 1995). According to Subasinghe (1992), antibiotics including tetracyclines, potentiated sulphonamides and chloramphenicol, are being used at present in the ornamental fish industry in Sri Lanka.

Among the 25 commonly used antibiotics evaluated in the present study (Table 12), aminoglycosides and various unclassified groups were found to be the most efficient in inhibiting majority of the bacterial isoltes. Antibiotic sensitivity pattern of different isolates indicated that the maximum bacterial isolates associated with disease conditions in marine ornamental fish were susceptible to gentamicin followed by streptomycin and OTC. In addition, nalidixic acid, ciprofloxacin, OTC, kanamicin, rifampicin, doxycycline and co-trimoxazole were effective to more than 75% of the total bacterial isolates checked. Gentamicin grouped under aminoglycosides inhibited the growth of 96.8% of the bacterial isolates. Gentamicin is considered as one of the important orally administered as well as injectable antibiotic compound (Stoskopf, 1993). Among 121 antibiotic agents tested, Austin *et al.* (1981) recorded gentamicin as one of the important compound, which possessed widest inhibitory spectrum towards *Alteromonas*, *Hyphomponas*, *Photobacterium* as well as *Vibrio* species.

Although compounds such as furanace, OTC, nifurprazine and sulphonamides-trimethoprim mixtures were advocated for controlling vibriosis infections (Austin *et al.*, 1981), very little information is available about the application of gentamicin as a wide spectrum antibiotic agent to marine ornamental fish associated bacterial pathogens.

Based on the antibiogram (Table 16) it was observed that the majority of the commonly used antibiotic compounds had a minimum of 50% sensitivity against most of the bacterial cultures tested In a study on the antibiogram of mangrove bacterial flora of Andaman, the bacterial isolates including *Aeromonas*, *Vibrio*, *Escherichia*, and *Enterobacter*, showed a minimum of 50% resistance against chloramphenicol (Shome *et al.*, 1995). In the present study, 75% of all the isolates were sensitive to chloramphenicol.

The *Serratia* species isolated and characterised in the present investigations was found to follow the general sensitivity pattern. Gentamicin

and ciprofloxacin with low MIC values of 4 µg/ml (Table 18) was inhibitory to the growth of the isolate. In addition, the isolate was sensitive to trimethoprim. In the experiments carried out by Baya *et al.* (1992) two strains of *S. marcescens* as well as *S. plymuthica* were reported to be sensitive to trimethoprim and oxolinic acid, although *S. marcescens* was resistant to most of the antibiotics tested in the experiments. Their study indicated that the potentiated sulphonamides in addition to macrolides and various unclassified groups were important in inhibiting the growth of these isolates. It was seen that both the strains displayed the same sensitivity pattern, being resistant to majority of the chemotherapeutic agents tested. Only the quinolones and the potentiated sulphonamides were highly effective against both the isolates. However, *S. plymuthica* strain was sensitive to many of the antimicrobial compounds. Oxolinic acid, flumequine and sulphamethoxazole were the only compounds to which the isolates were resistant. In another study by Nieto *et al.* (1990) Cholramphenicol, OTC, Streptomycin, flumequine, and oxolinic acid were reported to inhibit the same species isolated from rainbow trout. In addition, *S. plymuthica*, isolated from turbot was inhibited by Trimethoprim at 2.5µg in combination with sulphamethoxazole. The OTC at 30 I.U. was found to be growth inhibitory while with the present studies, OTC at the rate of 20µg/ml was recorded as the MIC value.

In the study by Vigneulle and Baudin Laurencin (1995) *S. liquefaciens* isolated from turbot showed sensitivity to all the isolates tested viz. chloramphenicol, trimethoprim with sulphamethoxole, flumequine, furanes, oxytetracycline and oxolinic acid. Another study by McIntosh and Austin (1990) indicated almost similar responses by the same species isolated from Atlantic salmon, *Salmo salar* with sensitivity to cholramphenicol, OTC in addition to Streptomycin, nalidixic acid neomycin and chlorteteracycline.

Torkildsen *et al.* (2000), reported that the MIC values of trimthoprim ranged from 0.5 to 16µg /ml when tested against bacteria associated with scallops. In the present study, the MIC value was 6.66 µg/ml

which may be comparable to the average value expressed by Torkildsen *et al.* (2000) for the same compound with marine bacteria comprising mainly *Vibrio*, *Aeromonas* and *Pseudomonas* isolated from scallops.

Studies by Kuo and Chung (1994) indicated that *A. hydrophila*, *E. tarda*, and *F.columnaris* were more sensitive to norfloxacine, flumequine, and oxolinic acid than to nalidixic acid, chloramphenicol, oxytetracycline and sulphamethazine. In their study, among the four tested quinolones, the minimum inhibition concentration of norfloxacine was the lowest, followed by flumequine, oxolinic acid and nalidixic acid.

Antiseptic compounds

The results of inhibitory activity of antiseptic compounds revealed that apart from formalin, malachite green and acriflavin were exhibiting maximum inhibitory activity towards the *S. marcescens* isolate (Table 19). In general, acriflavin as well as malachite green have been considered as potential chemotherapeutic antiseptic compounds, inhibiting the growth of microbes and hence widely recommended for use. Acriflavin at the rate of 5 ppm was added as a continuous treatment to ward off several diseases causing agents, including protozoa (Sniezko, 1978). However, according Leong (1993), acriflavin is used less in fish farming nations of the European Economic Community.

In Sri Lankan fish culture industry, use of chemicals such as formalin, Dipterex, copper sulphate and ferrous suphate have been reported by Subasinghe (1992). In the ornamental fish rearing,chemicals such as malachite green, potassium permanganate, methylene blue, formalin, copper sulphate are being used (Subasinghe, 1992).

According to Leong (1993) malachite green is widely used in fish farming in the industry in the European Economic Community. It was pointed out by Sindermann (1990) that chemicals such as malachite green may be

carcinogenic. The issues involved in the use of these compounds in aquaculture are many and compounds recommended for use are few. Majority of the problems involved are associated with food fish and their application in ornamental fish culture has not so far become a grave issue. However, with all the limitations, chemotherapy and chemoprophylaxis make significant contributions to tackle the disease problems (Sindermann, 1990).

Marine Natural Products

It was noticed that methanol extracts from several of the seaweeds and sponges were capable of acting as inhibitory compounds and the bioactive principles present in them were highly inhibitory against the tested pathogen (Table 20). The red seaweed, *Gracilaria corticata* (zone diameter-36mm) and sponge species, *Callyspongia* (zone diameter-25mm) and *Sigmodocea carnosa* (zone diameter-25mm) were found to be exhibiting high activity. Among seaweeds other than *Gracialaria corticata*, *Valanopsis pachyderma* was showing high inhibitory activity. All the three sponge isolates had moderate activity with zone diameters of 20 mm. Other studies conducted recently indicated similar results (Lipton, 2001). These results indicate that these extracts may be used as valuable management procedures against bacterial diseases.

In the recent past, influence of marine natural products of algal origin in combating bacterial disease has been initiated. Lustigman *et al.* (1992) have reported the effectiveness of antimicrobial compounds produced by 35 seaweed species of the New York coast against 3 species of bacteria. A preliminary extraction of the inhibitory substances was undertaken in their study. Porcile *et al.* (1991) discussed the effectiveness and host range of the active compounds from algae in relation to the aerobic Gram negative bacteria. The ecological aspects of algal antimicrobial agents and their interactions with reference marine aerobic Gram negative bacteria were also reported.

Antibiotic activity of 30 marine macroalgae against Gram negative bacteria and *Bacillus* was evaluated by Rao *et al.* (1991). Their results indicate that *Enteromorpha compressa*, *Cladophoropsis zoolingeri*, *Padina gymnospora*, *Sargassum wightii*, and *Gracilaria corticata* were active against Gram negative bacteria. The results of the present study, in which high inhibition was noted in *Gracilaria corticata* against the pathogenic isolate of *S. marcescens* confirms the earlier observations cited above.

The antibacterial properties of extracts of marine invertebrates including sponges were also investigated by a few researches. Wright *et al.* (1991) were able to obtain promising results on antibiotic and antifouling properties of marine invertebrate extracts. In the studies by Mary *et al.* (1994), eighteen bacterial isolates from five genera, *Aeromonas*, *Alcaligenes*, *Flavobacterium*, *Pseudomonas*, and *Vibrio*, isolated from biofilm associated with *Perna* sp. were tested for sensitivity to extracts from sponges, anemones, and a polychaete worm. The extracts of sponges, *Spirastrella inconstans* and *Spongia* officinalis, were found to be bacteriostatic. Antimicrobial assays showed that thin-layer chromatography fraction 6 of *S. inconstans* and fraction 7 of *S. officinanlis* inhibited the growth of the bacteria at an extract concentration equivalent to 10 mg original wet weight of tissue per disc.

Although experiments by Ilan *et al.* (1996) and Jung *et al.* (1995) proved that extracts of sponge species, have antibiotic role, further research on their role in controlling fish diseases are necessary so as to advocate them for use in aquaculture.

Immunoprophylactic studies

Vaccination of fish for the prevention of specific bacterial diseases of commercially reared fish species has had a specific and significant impact (Newman, 1993). It is well known that the management of bacterial diseases using antibiotics and antiseptic compounds has limited success due to frequent application and drug resistance problems. Though vaccination attempts were

reported for food fish, as far as marine ornamental fish are concerned, relatively less amount of work was carried out to make comparative accounts about the protective role of bacterins. Hence, based on the work on immunisation carried out in other marine fish culture, the present work may be evaluated.

Almost all of the vaccines available at present are bacterins or formalin-inactivated whole cell suspensions, some with adjuvants (Newman, 1993). Although, numerous fish vaccines have been developed, a few had only limited success (Adams *et al.*, 1999). Effective commercial vaccines were produced only for a few infectious diseases among salmonids and catfish such as enteric red mouth (ERM), vibriosis and furunculosis for salmonids and enteric septicaemia (ESC) vaccines for catfish. The other vaccines being tested including atypical *A. salmonicida*, *A. hydrophila*, *E. ictaluri*, *F. columnaris*, *Streptococcus* sp. and *Pasteurella piscicida*.

In the present study, fishes administered with bacterins of *S. marcescens* when challenged with the same live cells, gave a RPS of 75%, at 15th day of immunisation and 100% from 35th day onwards. This PRP was retained almost up to 50 days. The protection obtained by intraperitoneal injection of formalin-killed cells was the technique used to determine the role of immunisation in providing protection to fish, when challenged with the pathogenic isolate of *S. marcescens*. The results of immunoprophylactic experiments suggested that the percentage relative protection of experimental group of fish, which received intraperitoneal injection of bacterin, was substantially enhanced compared to those of control groups.

Not many studies were conducted in the past on the immunisation against infection with *Serratia* species. McIntosh and Austin (1990) reported the effectiveness of vaccination regimes of *S. liquefaciens* in Atlantic salmon, which was proved by challenge studies via intraperitoneal route in fish, vaccinated with whole cell as well as toxoid preparation. According to them, the immunised fish did not succumb to infection compared to the unvaccinated control, which

suffered 50% mortality. Similar results were obtained in the present study in the case of immunisation of *S. marcescens* bacterin in *Amphiprion* at 35th and 50th of immunisation.

In the study by McIntosh and Austin (1990) 0.4% (v/v) formalin was used for inactivation of whole cell vaccine, which was diluted to 1:10 with tap water and administered via bath for one minute followed by second bath 14 days later. The toxoid was prepared from ECP's and freeze-dried which was then resuspended in distilled water up to a concentration of 0.4mg protein per ml, for use. Toxoiding was achieved by addition of formalin to a final concentration of 0.4% (v/v) and administered via single intraperitoneal injection. Challenge was conducted 28 days later by intraperitoneal injection with 0.1ml of solution containing 10^6 cells. Results of challenge studies of their experiments were identical to those obtained in the study on immunisation of *Amphiprion* with *S. marcescens* bacterins, although administration of vaccine was done via intraperitoneal route in the present study where comparatively large durations of protection were established by using challenge studies.

It is already established that in order to administer an exact dose of vaccine, it is advantageous to inject the fish. *Enterococcus* bacterin ET-2 proved to be very effective when it was delivered by injection, the RPS values of which achieved by intraperitoneal injection route ranged from 89 to 100% (for 45-g turbot) and from 67 to 86% (for 150-g turbot), depending on the bacterial levels and time of experimental challenge. Moreover, this strong degree of protection lasted for at least 1 year (Toranzo *et al.*, 1995). The injection method of vaccination is particularly important in the case of marine ornamental fish, which command high market value.

One of the earlier bacterins developed for use in fish was to control vibriosis particularly in salmonids (Hayashi *et al.*, 1964; Fryer *et al.*, 1978). The results of these authors indicated that bacterins administered through injection were comparatively more effective. Many other authors, based

on injection immunisation studies documented similar results. Hence, in the present experiments also, intraperitoneal injection of bacterins at a rate equivalent to the infective dose was administered.

In the present study, formalin was used to kill the pathogen in order to prepare the bacterin. Formalin at the rate of 0.3% to 0.5% is usually used to prepare the killed bacterin (Fryer *et al.*, 1978). The concentration used in the present study was 0.3%, which brought about the killing the cells, without losing antigenic properties and imparted protection to the experimental group of fish. Studies by Qin and Pan (1996) showed that formalin killed vaccines of *V. vulnificus* gave rise to a satisfactory safety against experimental infection with the live pathogen.

Out of the different routes like immersion, oral and intraperitoneal injection, tried for vaccinating the flounder, *Paralchthyes olivaceus*, against *E. tarda*, the intraperitoneal injection gave high protective response, when formalin killed cells were used, where a RPS of 60% was obtained (Bang and Lee, 1995). In the present study in marine ornamental fish, use of formalin killed bacterin for immunisation gave more promising results with better relative percentage survival (RPS).

Immunisation studies on tilapia (*O. oreochromis*) against *Streptococcus iniae* using homplogous isolate (ARS 10) and heterologous isolate (ARS 60) by intraperitoneal and intramuscular injections as well as intramuscular immunisation with the combined ARS10 and ARS 60 vaccine, suggested that both intraperitoneal and intramuscular injections stimulate significant antibody titres (Klesius *et al.*, 2000). From these studies a comparison of intramuscular and intraperitoneal routes can be made. It was seen that, the intraperitoneal injection of the homologous isolate lead to a relative percentage survival value of 45.6% whereas the heterologous isolate led to 93.7% RPS. The intramuscular immunisation route led to 17.7% RPS

when homologous isolate was used for administration and in case of heterologous isolate,a RPS of 59.3% was observed (Klesius *et al.*, 2000).

In the above studies by Klesius *et al.* (2000), with homologous and heterologous isolates, intraperitoneal route was found to be giving better results and the same method was adopted in the current experiment with marine ornamental fish. However in the present study, multivalent vaccines were not used, since the species *S. marcescens* was frequently associated with lesions of marine ornamental fish and studies were carried out to determine the effect of vaccination against this particular pathogen.

In a study by Kawakami *et al.* (1997), fish vaccinated with the Lipopolysaccharide Chloroform killed cells (LPS-CKC) bacterin gave RPS of 60% or greater when challenged by the immersion method using three different live *P. piscicida* strains and the efficacy of this bacterin continued for at least 35 days after vaccination. In addition to providing protection against an artificial challenge, the LPS-CKC bacterin also provided protection against natural infection by *P. piscicida*. However, in the present study with chloroform-killed cells, the protection conferred from natural infection could not be estimated.

Similar studies have also proved that injection administration of killed cells are more advantageous in controlling bacterial infections. According to Agius *et al.* (1983), traditional vaccines against *V. anguillarum* comprising formalin killed cells or bacterial membrane products gave the best protection when administered intraperitoneally. It was seen that intraperitoneal vaccination resulted in 100% protection whereas oral method led to only 50-70% protection.

Sakai *et al.* (1986) reported that immersion and injection vaccination led to elimination of pathogen, on challenge with *V. anguillarum* via intraperitoneal route. When 10^5 to 10^6 cells were administered, the cells were eliminated within 72 hours from internal organs. In the case of fish vaccinated

by injection method, the rate of elimination of pathogen was faster. These authors also suggested that the cellular defense mechanisms played an important part in protection of invaders. The role of antigenic compound in protecting fish against several bacterial diseases has been reported. (Fugihara and Nakatani, 1971; Amend and Johnson, 1981; Akhlaghi, 1999).

In experiments using *V. anguillarum* bacterin by the immersion method, Tebbit and Goodrich (1983) reported that the PRP value of chum salmon was enhanced to more than 67% after challenging with intraperitoneal injection with live cells of the bacterin. Better results were documented by Greger and Goodrich (1999) where, in a short-term experimental trial, multivalent *Vibrio* vaccines provided 99% protection after 43 days of post vaccination challenge. They also documented that the immunity could be retained at one year to a level of 79%. It was observed that Atlantic salmon vaccinated with an oil adjuvanted-vaccine containing *V. viscosus, V. wodanis* and atypical *A. salmonicida* produced relative percentage survival of 97% when challenged intraperitoneally with *V. viscosus* (Greger and Goodrich, 1999). In the same study, rainbow trout challenged with *V. viscosus* at 52 and 362 days post-vaccination produced an RPS of 93% and 79%, indicating the long-term protection offered by the vaccine.

In the study by Klesius *et al.*(2000), the protection conferred by bacterin was evaluated at 30 days post immunisation with intraperitoneal injection method which gave RPS of 45.6%, while in the present study carried out in *Amphiprion*, protection conferred was 75%, 100% and 100% on 15, 35 and 50 days respectively. It was seen that the level of protection gained by the intraperitoneal route was remarkably high. In vaccination trials by intraperitoneal injection method, in Atlantic salmon against furunculosis, it was noticed that at sampling times of three weeks, six weeks and six months, a positive association between antibody levels and survival was found, (Midtlyng *et al.*, 1996). Although antibody titres were not evaluated in the present study after challenge

with live cells, it was observed that at 15, 35 and 50 days after immunisation, the protection conferred was high.

The RPS of hybrid striped bass challenged 35 days after vibriosis vaccination was 75.0% among those challenged by intraperitoneal injection with 3.51×10^5 cells/fish. However when the density of challenged cells was reduced to $1/10^{th}$, the RPS increased to 86.7% among hybrid striped bass (*Morone saxatilis* × *M. chrysops*) using commercial *Vibrio anguillarum*-*V. ordalii* bacterin (Rogers and Xu, 1992). With slightly higher concentration of cells used for challenge (10^5 cells per gram), the present study gave more promising results since the protection conferred at 35^{th} day of immunisation was 100%.

From the studies of Quentel and Ogier de Baulny (1995), it was found that intraperitoneal injection protected juvenile turbot during a challenge performed 1 month after a single immunisation against *V. anguillarum*. The relative percentage protection was the same irrespective of whether the vaccination was done 62, 76 or 104 days post hatching. It was also clear from from their studies that two months after the first immunisation the juveniles were still protected and RPS values were higher than those obtained 1 month after vaccination.

Hjeltnes *et al.* (1989), reported that fish vaccinated twice appeared to be better protected than fish vaccinated once and the degree of protection was dependent on the route of administration of the vaccine. Thus, studies were conducted against *V. salmonicida* among Atlantic salmon. In the present study, vaccination was carried out only once and no booster dose was given. However, the protection conferred was high which indicated that even single dose could effectively protect the fish up to 50^{th} day of immunisation.

It was reported by Eggset *et al.* (1999) that vaccination of Atlantic salmon at the stage of smoltification yielded high immunological protection and humoral immune response, which proves that the immunisation of fish at early

stages will serve as competent measures of protecting fish against bacterial diseases. Thus in the area of disease management of marine ornamental fish, the immunisation of hatchlings would be an ideal strategy to restrict the occurrence of diseases.

From the studies of the past as well as from the present experiments it is evident that in the area of management of bacterial diseases, the available options to restrict diseases are use of antibiotics, or other antimicrobial compounds. Immunoprophylaxis can be advantageous to commonly encountered bacterial isolates. This can form the best alternative to the undue use of chemotherapeutants and their consequent adverse effects. As stated by Rohovec *et al.* (1981) the immunisation has the advantage of improving the general health of the animal in addition to providing protection from specific pathogens. It also reduces the use of an dependance on drugs and chemicals (Busch, 1994). Recent authors have stressed on the use of immunisation as proven and accepted tool in aquatic health management and have recorded that use of vaccines, immunostimulants and adjuvants in fish culture offers a wide range of attractive methods for inducing and building up protection against diseases (Busch, 1994; Anderson, 1992).

SUMMARY

SUMMARY

The analysis of environmental factors in the marine aquarium showed that the microbial load as well as the trend of death in the aquarium is not affected significantly by any of the water quality parameters. This could have been due to the presence of an effectively functioning biological filter in the aquarium, which eliminated the build up of nitrogenous wastes thereby regulating the bacterial load and due to the continuous aeration provided in the system. However, it was seen from principal component analysis that, in the aquarium, three main factors contributed to general water chemistry, including water and atmospheric temperature, nitrite, dissolved oxygen and pH.

Based on the statistical analysis, it was inferred that one single factor may not affect the general health of the aquarium held fish. On the contrary, individual factor in association with the others affect the overall well being of the fish.

From observations on occurrence of mass mortality in the aquarium, it was seen that sudden imbalances in nutrient parameters following resetting of aquaria or other natural reasons usually result in mortality, involving almost all groups of fish. In addition, sudden variations, or higher than normal fluctuations of parameters such as microbial load, nitrate, nitrite and ammonia, to both high and low extremes resulted in mass mortality.

The virulent bacterial isolate which produced characteristic ulcerative lesions among some of the common ornamentals including *Amphiprion* was characterised as *Serratia marcescens* using standard biochemical tests and is reported for the first time from marine aquarium from India.

The isolate, *S. marcescens* was found to be frequently involved in infections of fish, mainly pomacentrids, acanthuids and chaetodontids leading to

lesions such as fin erosions and hemorrhagic areas on the lateral sides of body. Preliminary *in vitro* experiments and *in vivo* experiments proved the high proteolytic nature of the isolate.

The important flora of bacteria belonged to genera like *Flavobacterium* (27%), *Vibrio* (18%), *Pseudomonas* (16%), *Alcaligenes* (16%), *Acinetobacter* (11%) and members of Enterobactericeae (8%) family.

The generation time required of the selected pathogenic isolate, *S. marcescens* was estimated as 1.2 hours.

The pathogenicity of the bacterial cells was found to be due to the Extracellular Products, which was demonstrated, by the *in vitro* and *in vivo* studies. Infective dose for the live cells was found to be equivalent to 5.01×10^5 cells per fish or 1×10^5 cells per gram of fish.

The protein values of the ECP's produced by *S. marcescens* ranged from 260.7 µg/ml, 147 µg/ml and 130.9 µg/ml in the case of filtered ECP, ECP extracted by cellophane method with medium containing 0.5% sodium chloride and ECP extracted by cellophane method with medium containing 3.5% sodium chloride respectively.

Based on *in vitro* studies with the ECP it was found that the ECP was heat labile and the gelatinase and caesinase activity was reduced on heat treatment at 50^0C for 10 minutes.

The pathogenicity of ECP of *S. marcescens* was confirmed by the *in vivo* studies. An infective dose as low as 12.02 µg per fish was sufficient to bring about 50% mortality, which amounted to 2.67 µg per gram body weight of fish.

The molecular weight of the protein responsible for the pathogenicity of the isolate was estimated by the SDS-PAGE and was found that the major proteins responsible for pathology were manifested at 15, 18 and 24 hours of growth of the bacteria, that their molecular weights ranged from 11kDa to 100 kDa and that most of the proteins had weights higher than 40 kDa.

From the susceptibility pattern of the important bacterial isolates, it was observed that the major antibiotics, which were capable of inhibiting the growth of majority of the pathogenic isolates were aminoglycosides belonging to gentamicin, streptomycin, tetracycline, nalidixic acid, and quinolones like ciprofloxacin. Based on MIC studies with 7 selected antibiotics, it was found that the MIC values of the antibiotics for the selected pathogen, *S. marcescens ranged* from 4 µg per ml to 120 µg per ml and MBC values ranged from 10µg per ml to 200µg per ml.

Antiseptic compounds did not have much effect in controlling the pathogenic isolate, *S. marcescens*, which was proved by the *in vitro* studies with eight selected compounds. Except formalin, acriflavin and malachite green, no other compound had good activity against the isolate.

It was found from the present studies that marine natural compounds (extracts of seaweeds and sponges) could be good alternatives in controlling the growth of the selected pathogenic isolate, *S. marcescens*. Extracts of the sponges (*Sigmodocea carnosa*, *Callyspongia* and an unidentified one), as well as those of two seaweeds (*Gracilaria corticata* and *Valanopsis pachyderma*) were found to have excellent inhibitiory activity. Among these, *G. corticata* exhibited higher inhibition than the conventional antibiotics.

Studies on immunoprophylaxis with *S. marcescens* gave promising results. The Percentage Relative Protection of 100% was obtained after 35th and 50th day post immunisation proving the efficacy of immunoprophylaxis in controlling bacterial diseases.

REFERENCES

REFERENCES

*Abraham, E. P., Chain, E., Fletcher, C. M., Gardner, A. D., Heatly, N. G., Jennings, M. A. and Florey, H. W., 1941. Further observations on penicillin. *Lancet ii*: 177-189.

Adams, A., Thompson, K. D. and Roberts, R. J., 1999. Fish vaccines. In: vaccine manual- The production and quality control of veterinary vaccines for use in developing countries. FAO animal production and health series no. 35 (ed. Mowat, N. and Rweyemamu, M.), Daya publishing house, Delhi, 434 pp.

Agius, C., Horna, M. T. and Ward, P. D., 1983. Immunisation of rainbow trout, *Salmo gairdneri* Richardson against vibriosis: comparison of an extract antigen with whole cell bacterin by oral and intraperitoneal routes. *J. Fish Dis.*, 6: 129-134.

Akhlaghi, M., 1999. Passive immunisation of fish against vibriosis, comparison of intraperitoneal, oral and immersion routes. *Aquaculture,* 180: 191-205.

Alderman, D. J. and Smith, P., 2001. Development of draft protocols of standard reference methods for antimicrobial agent susceptibility testing of bacteria associated with fish diseases. *Aquaculture,* 196, 3-4: 211-243

Alexander, J. B., Bowers, A. and Shamshoom, S. M., 1981. Hyper osmotic infiltration of bacteria in to trout: route of entry and the fate of the infiltrated bacteria. *Develop. Biol. Standard.*, 49. 441-445.

*Allen, F. W. and McDaniel, E. C., 1937. A study of the relation of temperature to antibody production in cold-blooded animals. *J. Immunol.*, 32: 143-152.

Alsina, M. and Blanch, A. R., 1993. First isolation of *Flexibacter maritimus* from cultivated turbot, *Scophthalmus maximus*. *Bull. Eur. Assoc. Fish Pathol.*, 13: 157-160.

Alsina, M. and Blanch, A. R., 1994. Improvement and update of a set of keys for biochemical identification of *Vibrio* species. *J. Appl. Bacteriol.*, 77: 719-721.

Amandi, A., Hiu. S. F., Rohovec, J. S. and Fryer, J. L., 1982. Isolation and characterisation of *Edwardsiella trada* from fall Chinook salmon (*Oncorhynchus tshawytscha*). *Appl. Environ. Microbiol.*, 43: 1380-1384.

Amend, D. F., 1981. Potency testing of fish vaccines. *Develop. Biol. Standard.*, 49:447-454.

Amend, D. F., Antipa, R. and Kerstetter, T. H., 1980. Increase in ocean survival

of freely migrating steelheads vaccinated against *Vibrio anguillarum*. *Trans. Am. Fish. Soc.*, 109: 287-289.

Amend, D. F. and Fender, D. C., 1976. Uptake of bovine serum albumin by rainbow trout from hyper osmotic solutions: a model for vaccinating fish. *Science*, 192: 793-794.

Amend, D. F. and Johnson, K. A., 1981. Current status and future needs of *Vibrio anguillarum* bacterins. *Develop. Biol. Standard.*, 49: 403-417.

Amend, D. F., and Ross, A. J., 1970. Virucidal activity of two iodophores to salmonid vaccines. *J. Fish Res. Bd. Canada*, 19: 61-65.

Anderson, D. P., 1988. Fish vaccination effectiveness monitored by *in vitro* culture. Aquaculture International Congress and Exposition, Vancouver, Canada, 52pp.

Anderson, D. P., 1992. Immunostimulants, adjuvants, and vaccine carriers in fish: Applications to aquaculture. *Annu. Rev. Fish Dis.*, 2: 281-307.

Anderson, D. P. and Nelson, J. R., 1974. Comparison of protection in rainbow trout inoculated with and fed Hagerman redmouth bacterins. *J. Fish Res. Bd. Canada*, 31: 2, 214-216.

*Anderson, J. I. W. and Conroy D. A. 1970. *Vibrio* disease in marine fishes. In: symposium on diseases of fishes and shellfishes (ed. Sniezko, S. F.). *Trans. Am. Fish. Soc.*, Spec. Pub., 5: 266-273.

Anon, 1998. New generation won over. Fish farming international, 27, 7:16.

Anon, 2001. Banned antibiotics: Notification by the Marine Products Exports Development Authority. Published in The Hindu. November 14, 2001.

Ansary, A., Haneef, R. M., Torres, L. and Yadav, M., 1992. Plasmids and antibiotic resistance in *Aeromonas hydrophila* isolated in Malaysia from healthy and diseased fish. *J. Fish Dis.*, 15: 191-196.

Antipa, R., Gould, R. and Amend, D. F., 1977. Immunisation of Pacific salmon: comparison of intraperitoneal injection and hyperosmotic infiltration of *Vibrio anguillarum* and *Aeromonas salmonicida* bacterins. *J. Fish. Res. Bd Canada*, 34: 203-208.

Aoki, T., 1999. Motile aeromonads (*Aeromonas hydrophila*). In: Fish Diseases and Disorders, Volume 3, Viral, Bacterial and Fungal Infections (ed. Woo, P. T. K. and Bruno. D. W.). CABI publishing, New York, pp. 427-453.

*Arakawa, C. K. and Fryer, J. L., 1984. Isolation and characterisation of a new subspecies of *Mycobacterium chelonei* infections for salmonid fish. *Helgoländer Meeresuntersuchungen*, 37: 329-342.

Arvedlund, M., McCormick, M. I. and Ainsworth, T., 2000. Effects of photoperiod on growth of larvae and juveniles of the anemone fish *Amphiprion melanopus*. Naga, The ICLARM Quarterly, 23, 2, April-June 2000, pp 18-23.

Austin, B., 1982. Taxonomy of bacteria isolated from a coastal marine fish-rearing unit. *J. Appl. Bacteriol.*, 53: 253-268.

Austin, B., 1983. Bacterial microflora associated with a coastal marine fish-rearing unit. *Journal of the Marine Biological Association* (UK), 63: 583-592.

*Austin, B., 1984. The control of bacterial fish diseases by antimicrobial compounds. In: Antimicrobials and Agriculture, Benefits and Malefits (ed. Woodbine, M.). Butterworths, London, pp.255-268.

Austin, B. 1985. Chemotherapy of bacterial fish diseases. In: fish and shellfish pathology (ed. Ellis, A. E.). Academic Press, London, pp. 19-26.

*Austin, B., Allen, D. A., Zachary, A., Belas, M. R. and Colwell, R. R., 1979a. Ecology and taxonomy of bacteria attaching to wood surfaces in a tropical harbor. *Can. J. Microbiol.*, 25: 446-461.

Austin, B. and Al-Zaharani, A. M. J., 1988. The effect of antimicrobial compounds on the gastro intestinal microflora of rainbow trout, *Salmo gairdneri* Richardson, *J. Fish Biol.*, 33: 1-14.

Austin, B. and Austin, D. A., 1987. Bacterial fish pathogens. Disease in farmed and wild fish, Ellis Horwood Ltd., Chichester, 364 pp.

Austin, B. and Austin, D. A., 1989. Methods for the microbiological examination of fish and shellfish. Ellis Horwood Ltd., Chichester, 317pp.

Austin, B., Colwell, R. R., Garges, S., Harding, L., Simidu, U. and Taga, N., 1979b. Comparative study of the aerobic heterotrophic bacterial flora of Chesapeake Bay and Tokyo Bay. *Appl. Environ. Microbiol.*, 37: 704-714.

Austin, B. and Gibb, A., 1993. Emerging bacterial fish pathogens and their likely significance for aquaculture. Proceedings of the first international symposium on aquaculture technology and investment opportunities. Ministry of agriculture and water, Riyadh, Saudi Arabia, pp. 410-425.

Austin, B., Morgan, D. A. and Alderman, D J., 1981. Comparison of antimicrobial agents for control of vibriosis in marine fish. *Aquaculture*, 26, 1-12.

Austin, B, Stewart Tull, D. E. S., Dennis, P. J. and Godfrey, A. F., (eds.) 1999. The effects of pollution on fish health. *J. Appl. Microbiol.*, 85: 234-242.

Austin, B. and Stobie, M., 1992. Recovery of *Serratia plymuthica* and presumptive *Pseudomonas pseudoalcaligenes* from skin lesions in

rainbow trout, *Oncorhynchus mykiss*, (Walbaum), otherwise infected with redmouth. *J. Fish Dis.*, 15: 541-543.

Avault, J. W., Jr., 1997. Prevention of diseases, some fundamentals reviewed. *Aquacult. Mag.*, 23, 2: 78-83.

*Bain, N. and Shewan, J. M. 1968. Identification of *Aeromonas*, *Vibrio* and related organisms. In: Identification methods for Microbiologists, Part B (ed. Gibbs, B. M. and Shapton, D. A.). Soc. Appl. Bacteriol. Tech. Ser., 2: 79-84.

Balebona, M. C., Zorilla, I., Moriñigo, M. A. and Borrego, J. J., 1998. Survey of bacterial pathologies affecting farmed gilt-head sea bream (*Sparus aurata* L.) in southwestern Spain from 1990 to 1996. *Aquculture.*, 166, 1-2: 19-35.

Bang, J. D. and Lee, J. S., 1995. Protective effectiveness to edwardsiellosis of olive flounder, *Paralichthys olivaceus* on *Edwardsiella tarda* bacterins. Bull. Natl. Fish. Res. Dev. Agency, Korea, 50: 67-76.

Barrow, G. I. and Feltham, R. K. A., 1993. Cowan and Steel's manual for the identification of medical bacteria, 3rd edition. Cambridge University Press. 331 pp.

*Bauer, A. W., Kirby, W. M. M., Sherris, J. C. and Turck, M., 1966. Antibiotic susceptibility testing by a standardised single disc method. *Am. J. Clin. Pathol.*, 45: 493-496.

Baumann, L., Baumann, P., Mandel, M. and Allen, R. D., 1972. Taxonomy of aerobic marine bacteria. *J. Bacteriol.*, 110: 402-409.

Baxa, D. V., Kawai, K. and Kusuda, R., 1986. Characteristics of gliding bacteria isolated from diseased cultured flounder *Paralicthys olivaceus*. *Fish Pathol.*, 21: 351-258.

*Baxa, D. V., Kawai, K. and Kusuda, R., 1988. *In vitro* and *in vivo* activities of *Flexibacter maritimus* toxins. Reports of Usa Marine Biology Institute. Kochi University, 10: 1-8.

Baya, A. M., Toranzo, A. E., Lupiani, B., Santos, Y. and Hetrick, F. M., 1992. *Serratia marcescens*: a potential pathogen of fish. *J. Fish Dis.*, 15: 15-26.

Bell, G. R., 1961. Two epidemics of apparent kidney disease in cultured pink salmon (*Oncorhynchus gorbuscha*). *J. Fish Res. Bd. Canada.*, 18: 559-562.

Bell G. R., Hoskins G. E. and Hodgkiss, W., 1971. Aspects of characterisation, identification and ecology of the bacterial flora associated with the surface of the stream-incubating Pacific salmon (*Oncorhynchus*) eggs. *J. Fish Res. Bd. Canada.*, 28: 1511-1525.

Benediktsdóttir, E., Helgason, S. and Sigurjónsdóttir, H., 1998. *Vibrio* spp. isolated from salmonids with shallow skin lesions and reared at low temperature. *J. Fish Dis.*, 21: 19-28.

Bernardet, J. F., Campell, A. C. and Buswell, J. A., 1990. *Felxibacter maritimus* is the agent of black patch necrosis in Dover sole in Scotland. *Dis. Aquat. Org.*, 8: 233-237.

Bernoth, E-V and Mark St. J. Krane, 1995. Viral diseases of aquarium fish. Seminars in Avian and Exotic Pet Medicine, 4, 2, 103-110.

Bertolini, J. M. and Rohovec, J. S., 1992. Electrophoretic detection of proteases from different *Flexibacter columnaris strains* and assessment of their variability. *Dis. Aquat. Org.*, 12, 2: 121-128.

Bertolini, J. M., Wakabayashi, H., Vatral, V. G., Whipple, M. J. and Rohovec, J. S., 1994. Electrophoretic detection of proteases from selected strains of *Flexibacter psychrophilus* and the assessment of their variability. *J. Aquat. Anim. Health,* 6: 224-233.

*Birnbaum, N. G., 1998. Licensing and regulation of veterinary biologics for fish in the United States. Nutrition and Technical Development of Aquaculture. (ed. Howell, W. H., Keller, B. J., Park, P. K., McVey, J. P., Takayanagi, K., Uekita, Y.) New Hampshire University, Durham, USA, Sea Grant Program, pp. 33-38

*Bisset, K. A., 1948. The effect of temperature upon antibody production in cold-blooded vertebrates. *J. Pathol. Bacteriol.*, 60: 87-92.

Boira, R. A., 1996. *Hydrophila* group aeromonads in environmental waters. Culture, 17: 1-4.

*Bondi, A., Spaulding, E. H., Smith, D. E. and Dietz, C. C., 1947. Routine method for rapid determination of susceptibility to penicillin and other antibiotics. *Am. J. med. Sci.*, 213: 221-225.

Bordas, M. A., Balebona, M. C., Zorrilla, I., Borrego, J. J. and Morinigo, M. A., 1996. Kinetics of adhesion of selected fish-pathogenic Vibrio strains to skin mucus of gilt-head sea bream (*Sparus aurata* L.). *Appl. Environ. Microbiol.*, 62, 10: 3650-3654.

Bosakowski, T. and Wagner, E. J., 1994. A survey of trout fin erosion, water quality, and rearing conditions at state fish hatcheries in Utah. *J. World Aquacult. Soc.*, 25, 2: 308-316.

*Boulanger, Y., Lallier, R. and Cousineau, G., 1977. Isolation of enterotoxigenic *Aeromonas* from fish. *Can. J. Microbiol.*, 23: 1161-1164.

*Braaten, B. and Hektoen, H., 1991. The environmental impact of aquaculture. In: Fish health management in Asia pacific. Report on a regional study and workshop on fish disease and fish health management. Asian Development Bank, Manila Philippines, Network of Aquaculture Centres in Asia, Bangkok, Thailand, Manila, Philippines ADB, 1: 469- 524.

Brock, J. A., LeaMaster, B. R., Lee and Cheng Sheng, 1993. An overview of pathogens and diseases in marine finfish hatcheries in Hawaii with comments on strategies for health management and disease prevention. In: Finfish hatchery in Asia. Proceedings of finfish hatchery in Asia ' 91. (ed. Lee, C. S., Su, M. S., Liao, I. Chiu). Keelung Taiwan Tungkang Marine Laboratory, TFRI, 3: 211-238.

Brown, A. L., 1978. Immunity and practical vaccine development. *Mar. Fish. Rev.*, 40, 3: 2-5.

Bruno, D. W., 1986. Histopathology of bacterial kidney disease in laboratory infected rainbow trout, *Salmo gairdneri* (Richardson) and Atlantic salmon, *Salmo salar*, L., with reference to naturally infected fish. *J. Fish Dis.*, 9: 52-537.

*Bry, C., 1988. Stress in cultivated fish: Possible causes, biological reactions, preventive measures. *Recl. Med. Vet.*, Ec., Alfort, Paris., 164, 10: 863-869.

*Bucke, 1980. Experimental and naturally occurring furunculosis in various fish species: a comparative study. In: Fish Diseases, (ed. Ahne, W.) Springer-Verlag, Berlin, pp. 82-86.

*Buggs, C. W., Bronstein, B., Hirschfeld, J. W. and Pilling, M. A., 1946. *In vitro* action of streptomycin on bacteria. *J. Am. med. Ass.*, 130: 64-67.

Bullock, G. L., 1961. A schematic outline for the presumptive identification of bacterial diseases of fish. *Prog. Fish-Cult.*, 23: 147-150.

Bullock G. L. and McLaughlin, J. J. A., 1970. Advances in knowledge concerning bacteria pathogenic to fishes. (1954-1968). In: A symposium on diseases of fishes and shellfishes (ed. Sniezko, S. F.). American Fisheries Society, Washington DC, Special Publication no 5: 231-242.

Bullock G. L. and Stuckey, H. M. and Shotts, E. B., 1978. Enteric redmouth bacterium: comparison of isolates from different geographic regions. *J. Fish Dis.*, 1, 361-366.

Bunch, E. C. and Bejerano, I, 1997. The effect of environmental factors on the susceptibility of hybrid tilapia *Oreochromis niloticus* x Oreochromis *aureus* to Streptococcosis. *Isr. J. Aquacult.* Bamidgeh, 49, 2: 67- 76.

*Busch, R. A., 1994. Immunisation as a tool in fish health management. International symposium on aquatic animal health: program and abstracts. Davis, CA USA Univ. of California, school of veterinary medicine, pp. p. II-3.

Cahill, M. M., 1990 a. Bacterial flora of fishes: a review. *Microb. Ecol.*, 19: 21-41.

Cahill, M. M., 1990 b. Virulence factors in motile *Aeromonas* species. *J. Appl. Bacteriol.*, 69: 1-16.

Candan, A., Kucuker, M. and Karatas, S. 1995. Motile Aeromonad septicaemia in *Salmo salar* cultured in the Black Sea in Turkey. *Bull. Eur. Assoc. Fish Pathol.*, 15: 195-196.

Cardella, M. A. and Eimers, M. E., 1990. Safety and potency testing of federally licensed fish bacterins. *J. Aquat. Anim. Health*, 2, 1: 49-55.

Cerdà cuéllar, M., Rosselló Mora R. A., Lalucat J., Jofre, J. and Et blanch, A., 1997. *Vibrio scophthalmi* sp. nov., a new species from turbot (*Scophthalmus maximus*). *Int. J. Syst. Bacteriol.*, 47: 58-61.

Chapman, F. A., Fitz-Coy, S. A., Thunberg, E. M. and Adams, C. M., 1997. United States of America trade in ornamental fish. *J. World Aquacult. Soc.*, 28, 1: 1-10.

Chen, S-C., Adams, A. and Richards, R. H. 1997. Extracellular products from *Mycobacterium* spp. in fish. *J. Fish Dis.*, 20: 19-25.

*Chen,Y., Qian, D., Shen, Z., Shen, J., Yin, W. and Zhang, N., 1996. Preparation of bacterin for fish bacterial septicemia. *Shuichan Xuebao*, 20, 2: 125-131

Collins, C. H. and Lyne, P. M., 1976. Microbiological methods, 4th Edition. Butterworths, London.

*Colt, J., Bouck, G. and Fidler, L., 1987. Review of current literature and research on gas supersaturation and gas bubble trauma: Special publication number 1, Fish Factory, Davis, CA (USA), 53 pp.

Colwell, R. R. 1962. The bacterial flora of Puget Sound fish. *J. Appl. Bacteriol.*, 28: 147-158.

*Colwell, R. R. and Grimes, D. J., 1984. *Vibrio* diseases of marine fish populations. *Holgoland Meerisunters.*, 37: 265-287.

Colwell, R. R. and Liston, J., 1960. Taxonomic relationships among the pseudomonads. *J. Bacteriol.*, 82: 1-14.

*Conroy, D. A., 1961.Estudio *in vitro* de la accion de la Kanamycina sobre bacterias patogenas paralos peces. *Microbiol. Espanolo*, 14, 147-155.

*Conroy, D. A., 1984. Agents: Bacteria. In: "Diseases of Marine Animals" (ed. Kinne, O.), Vol. 4, Part 1, Biol. Anstalt Helgol., Hamburg, Federal Republic of Germany, pp. 48-88.

*Cordova, S. M., Auro de O, A. and De Buen de A, N., 1996. Histopathological lesions produced in the tilapia (*Oreochromis* sp.) in experimental aquarium culture. *Vet. Mex.*, 27, 2: 143-148.

*Cossins, A. R., 1983. The adaptation of membrane structure and function to changes in temperature. In: Cellular Acclimation to Environmental Change (ed. Cossins A. R. and Sheterline, P.). Cambridge University Press, Cambridge, pp. 3-32.

*Crosby, M. D., 1996. Fish health status of aquaculture recirculating systems in Virginia: Three years of casework. Aquacultural Engineering Society Proceedings II: Successes and Failures in Commercial Recirculating *Aquaculture*, 2: 611- 615.

Croy, T. R. and Amend, D. F., 1977. Immunisation of sockeye salmon (*Oncorhynchus nerka*) against vibriosis using hyperosmotic infiltration technique. *Aquaculture*, 12: 317-325.

*Dalsgaard, A., Glerup, P., Hoybye, L. L., Paarup, A. M., Meza, R., Bernal, M., Shimada, T. and Taylor, D. N., 1997. *Vibrio furnissii* isolated from humans in Peru: A possible human pathogen? *Epidemiol. Infect.*, 119, 2: 143-149.

Daoust, P. Y. and Ferguson, H. W., 1984. The pathology of chronic ammonia toxicity in rainbow trout, *Salmo gairdneri* Richardson. *J. Fish Dis.*, 7, 199-205.

Dawes, J., 1998. International Experience In Ornamental Marine Species Management- Part 1: Perspectives. In: Management Strategies for the Marine Ornate Species of the Gulf of California, La Paz, Baja California Sur, Mexico.

DeFigueiredo, J. and Plumb, J. A., 1977. Virulence of different isolates of *Aeromonas hydrophila* in channel catfish. *Aquaculture*, 11: 349-354.

Diggles, B. K., Carson, J., Hine, P. M. Hickman, R. W. and Tait, M. J., 2000. *Vibrio* species associated with mortalities in hatchery reared turbot (*Colistium nudipinnis*) and brill (*C. guntheri*) in New Zealand. *Aquaculture*, 183: 1-12.

*Dixon, B. A., 1991. Antibiotic resistance of bacterial fish pathogens. LARVI '91. (ed. Lavens, P., Sorgeloos, P., Jaspers, E., Ollevier, F.). Fish and Crustacean Larviculture Symposium, Gent, Belgium, 15: 419.

Doukas, V., Athanassopoulaou, F. Karagouni, E. and Dotsika, E., 1998. *Aeromonas hydrophila* infection in cultured sea bass, *Dicentrarchus labrax*

L., and *Puntazzo puntazzo Cuvier* from the Aegean Sea. *J. Fish Dis.*, 21: 317-320.

*Duff, D. C. B., 1942. The oral immunisation of trout against *Bacterium salmonicida*. *J. Immunol.*, 44: 87-94.

*Earp, B. J., Ellis, C. H. and Ordal, E. J., 1953. Kidney disease in young salmon. Spec. Sci. Rep. Wash. Dep. Fish., 1, 73 pp.

Eggset, G., Mortensen, A. and Løken S., 1999. Vaccination of Atlantic salmon (*Salmo salar* L.) before and during smoltification, effects on smoltification and immunological protection. *Aquaculture*, 170: 101-112.

Egidius, E. C. and Anderson, K., 1979. Bath immunisation-a practical and non-stressing method of vaccinating sea farmed rainbow trout, *Salmo gairdneri* Richardson, against vibriosis. *J. Fish Dis.* 2: 405-410.

Ellis, A. E., 1988. General principles of vaccination. In: fish vaccination. (ed. Ellis, A. E.). Academic Press, pp.1-19.

Ellis, A. E., 1991. An appraisal of the extracellular toxins of *Aeromonas salmonicida* ssp. *salmonicida*. *J. Fish Dis.*, 14: 265-267.

Ellis, A. E., Burrows, A. S. and Stapleton, K. J. 1988. Lack of relationship between virulence of *Aeromonas salmonicida* and the putative virulence factors: A –layer extracellular proteases, and extracellular hemolysins. *J. Fish Dis.*., 11: 309-323.

Ellis, A. E. and Grisley, M. S., 1985. Serum antiproteases of salmonids: Studies on the inhibition of trypsin and the proteolytic activity of *Aeromonas salmonicida* extracellular products. *In*: Fish and shellfish pathology. (ed. Ellis, A. E.), Academic Press, London, pp. 85-96.

Ellis, A. E., Hastings, T. S. and Munro, A. L. S., 1981. The role of *Aeromonas salmonicida* extracellular products in the pathology of furunculosis. *J. Fish Dis.*, 4: 41-51.

Ellis, R. W., Novotny, A. J. and Harrell, L. W., 1978. Case report of kidney disease in a wild Chinook salmon (*Oncorhynchus tshawytscha*) in the sea. *J. Wildl. Dis.*, 14, 121-123.

*Emmens, C. W., 1995. Marine aquaria and miniature reefs. TFH Publ. Inc. 320 pp.

Endo, T., Sakuma, M., Tanaka, H., Ogishima, K., Hara, T., Ohshima, S. and Sato, Y., 1973. Application of oxolinic acid as a chemotherapeutic agent against infectious diseases in fishes. II. Explanation of chemotherapeutic effects by whole body autoradiography. *Bull. Jpn. Soc. Sci. Fish.*, 39, 2: 173-177.

Esteve, C., Amaro, C., Biosca, E. G. and Garay, E., 1995. Biochemical and toxigenic properties of *Vibrio furnissii* isolated from a European eel farm. *Aquaculture*, 132, 1-2: 81-90.

Evans, J. J., Shoemaker, C. A. and Klesius, P. H., 2000. Experimental infection of hybrid striped bass (*Morone chrysops*× *Morone saxatilis*) and tilapia (*Oreochromis niloticus*) by nares inoculation. *Aquaculture*, 189: 197-210.

*Ewing, W. H., Davis, B. R. and Reavis, R. W., 1959. Studies on the *Serratia* group. CDC Laboratory Manual, Communicable Diseases Centre, Atlanta, Ga.

*Finegold, S. M. and Martin, W. J., 1982. Bailey and Scott's Diagnostic microbiology. The C. V. Mosby Comapany, Philadelphia and Toronto.

*Fleming, A., 1929. On the antibacterial action of cultures of a *Penicillium* with special reference to their use in the isolation of *B. influenzae*. *British J. Exp. Pathol.*, 10: 226-236.

*Fleming, A., 1942. *In vitro* tests of penicillin potency. *Lancet i*: 732-733.

Fouz, B., Barja, J. L., Amaro, C., Rivas, C. and Toranzo, A. E., 1993. Toxicity of the extracellular products of *Vibrio damsela* isolated from diseased fish. *Curr. Microbiol.*, 27, 6: 341-347

*Frank, P. F., Wilkox, C. and Finland, M., 1950. *In vitro* sensitivity of coliform bacilli to 7 antibiotics (penicillin, streptomycin, bacitracin, polymyxin, aerosporin, aureomycin and chloromycetin). *J. Lab. clin. Med.*, 35: 188-204.

Freda, J. and McDonald, D. G., 1988. Physiological correlates of interspecific variation in acid tolerance in fish. *J. Exp. Biol.*, 136: 243-258.

*Fryer, J. L., Nelson, J. S. and Garrison, R. L., 1972. Vibriosis in fish. In: Progress in fishery and food science (ed. Moore, R. W.), University of Washington publications, 5: 129-133.

*Fryer, J. L. and Pilcher, K. S., 1974. Effects of temperature on diseases of salmonid fishes. EPA Report 660/3-73-020. 112 pp.

Fryer, J. L., Rohovec, J. S. and Garrison, R. L., 1978. Immunisation of salmonids for control of vibriosis. *Mar. Fish Rev.*, 40, 3: 20-23.

Fugihara, M. P. and Nakatani, R. E., 1971. Antibody production and immune response of rainbow trout and Coho salmon to *Chondrococcus columnaris* *J. Fish Res. Bd. Canada.*, 28, 9: 1253-1258.

Fukuda, Y. and Kusuda, R., 1985. Vaccination of yellowtail against pseudotuberculosis. *Gyobyo Kenkyu*, 20: 421- 425.

Fyfe, L., Finley. A., Coleman, G. and Munro, A. L. S., 1986. A study of the pathological effect of isolated *Aeromonas salmonicida* extracellular proteases on Atlantic salmon, *Salmo salar* L. *J. Fish Dis.*, 9: 403-409.

*Gelev, I., Gelev, E., Steigerwalt, A. G., Carter, G. P. and Brenner, D. J., 1990. Identification of the bacterium associated with haemorrhagic septicaemia in rainbow trout as *Hafnia alvei*. *Res. Microbio.*, 141: 573-576.

*Ghittino, P. and Prearo, M., 1992. Report of Streptococcosis in rainbow trout (*Oncorhynchus mykiss*) in Italy: Preliminary note. *Boll. Soc. Ital. Patol. Ittica*, 8: 4-9.

Giavenni, R., Finazzi, M., Poli, G. and Grimaldi, E., 1980. Tuberculosis in marine tropical fishes in an aquarium. *J. Wildl. Dis.*, 16: 161-168.

Gillespie, N. C. and Macrae, I. C., 1975. The bacterial flora from some Queensland fish and its ability to cause spoilage. *J. Appl. Bacteriol.*, 31: 91-100.

Gilmour, A., McCallum, M. F. and Allan, M. C., 1975. Antibiotic sensitivity of bacteria isolated from the canned eggs of the California brine shrimp (*Artemia salina*) *Aquaculture*, 6, 221-226.

Gomes, L. A., 1996. Can marine ornamental fish be farmed? Infofish International, 3/96, 27-32.

Gordin, H., Krom, M., Neori, A., Popper, D., Porter, C. and Shpigel, M., 1990. Intensive integrated seawater fishponds: Fish growth and water quality. In: Research in modern aquaculture. Proceedings of the 3rd status seminar, (ed. Rosenthal, H., Sarig, S.). Tiberius, Israel, 11 pp. 45-64.

*Gould, J. C. and Bowie, J. H., 1952. The determination of bacterial sensitivity to antibiotics. Edinburgh Medical Journal, 59: 178-199.

Gould, R. W., Antipa, R. and Amend, D. F., 1979. Immersion vaccination of sockeye salmon (*Oncorhynchus nerka*) with two pathogenic strains of *Vibrio anguillarum*. *J. Fish Res. Bd. Canada*, 36: 222-225.

Gould, R. W., O'Leary, P. J., Garrison, R. L., Ronovec, J. S. and Fryer, J. L., 1978. Spray vaccination: a method for immunisation of fish. *Fish Path.*, 13: 63-68.

Gram, L. and Huss, H. H., 1996. Microbiological spoilage of fish and fish products. *Int. J. Food Microbiol.*, 33, 1: 121-137.

Gratzek, J. B., Shotts, E. B. and Blue, J. L., 1978. Ornamental fish: diseases and problems. *Mar. Fish. Rev.*, 40, 3: 58-60.

Greger, E. and Goodrich, T., 1999. Vaccine development for winter ulcer disease, *Vibrio viscosus*, in Atlantic salmon, *Salmo salar*, L. *J. Fish Dis.*, 22: 193-199.

Gudmundsdóttir, B. K., Johnsdóttir, H., Steinthorsedótir, R., Magnadóttir, B. and Budmundsdóttir, S., 1997. Survival and humoral antibody response of Atlantic salmon, *Salmo salar* L., vaccinated against *Aeromonas salmonicida* spp. achromogenes. *J. Fish Dis.*, 20: 351-360.

*Gutenberger, S. K., Duimstra, J. R., Rohovec, J. S. and Fryer, J. L., 1991. Inracellular survival of *Renibacterium salmoninarum* in trout macrphages. Abstract. In: 14th annual AFS/FHS Meeting and the 32nd Western Fish Disease Conference, Newport, Oregon, July 31st -Aug 3rd.

*Gutsell, J., 1946. Sulfa drugs and the treatment of furunculosis in trout. *Science*, 104: 85-86.

Hackett, J. L., Lynch, W. H., Paterson, W. D. and Coombs, D. H., 1984. Extracellular protease, extracellular hemolysis, and virulence in *Aeromonas salmonicida*. *Can. J. Fish. Aquat. Sci.*, 41: 1354-1360.

Haenen, O. L. M. and Davidse, A., 2001.First isolation and pathogenicity studies with *Pseudomonas anguilliseptica* from diseased European eel *Anguilla anguilla* (L.) in The Netherlands. *Aquaculture*, 196, 1-2: 27-36.

Hameed, A. S. S. and Rao, P. V, 1993. Studies on the pathobiology of penaeid larvae and post larvae. Mariculture research under the postgraduate programme in mariculture. Part 5, (ed. Rengarajan, K., Noble, A., Prathibha, P., Kripa, V., Sridhar, N., Zakhariah, M.). CMFRI, Cochin, India. 56: 105-107.

Handlinger, J. Soltani, M. and Percival, S. 1997. The pathology of *Flexibacter maritimus* in aquaculture species in Tasmania, Australia. *J. Fish Dis.*, 20: 159-168.

Handy, R. D. and Poxton, M. G., 1993. Nitrogen pollution in mariculture: Toxicity and excretion of nitrogenous compounds by marine fish. *Rev. Fish Biol. Fish.*, 3, 3: 205- 241.

Hansen, G. H. and Olafsen, J. A., 1989. Bacterial colonisation of cod (*Gadus morhua* L.) and halibut (*Hippoglossus hippoglossus*) eggs in marine aquaculture. *Appl. Environ. Microbiol.*, 55, 6, 1435-1446.

Hanson, L. A. and Grizzle, J. M., 1985. Nitrite induced predisposition of channel catfish to bacterial disease. *Prog. Fish-Cult.*, 47: 98-101.

Hargis, W. J., Jr., 1991. Disorders of the eye in finfish. *Annu. Rev. Fish Dis.*, 1: 95-117.

Harrell, L. W., 1978. Vibriosis and current and current vaccination procedures in Puget Sound, Washington. *Mar. Fish. Rev.*, 40: 24-25.

Harrell, L. W., 1979. Immunisation of fisheries in world mariculture: a review. *Proc. World Maricult. Soc.*, 10: 534-544.

Hatai, K., Lawhavinit, O., Kubota, S. S., Toda, K. and Suzuki, N., 1988. Pathogenicity of *Mycobacterium* sp. isolated from pjarrey, *Odonthestes bonariensis*. *Fish Pathol.*, 23: 155-159.

*Hayashi, K., Kobayashi, S., Kamata, T. and Ozaki, H., 1964. Studies on the *Vibrio* disease of rainbow trout (*Salmo gairdneri* irideus). II Prophylactic vaccination against the *Vibrio*-disease. *J. Fac. Fish. Prefect. Univ. Mie* (Japan), 6: 181-191.

Hazen, T. C., Fliermans, C. B., Hirsh, R. P. and Esch, G. W, 1974. Prevalence and distribution of *Aeromonas hydrophila* in the United States. *Appl. Environ. Microbiol.*, 36: 731-738.

Hedrick, R. P., McDowell, T. and Groff, J., 1987. Mycobacteriosis in cultured striped bass from California. *J. Wildl. Dis.*, 23: 391-395.

Heo, G. J., Wakabayashi, H. and Watabe, S., 1990. Purification and characterisation of pili from *Flavobacterium branchiophila*. *Fish Pathol.*, 25: 21-27.

*Heslinga, G. A., 1996. Sustainable aquaculture in the marine aquarium industry. In: Proceedings of the PACON Conference on Sustainable Aquaculture ' 95, Honolulu, HI (USA). p. 174

Hiatt, W. N., 1998. The new right now!™ 24 hour entirely aerobic nitrification cycle. *J. Shellfish Res.*, 17, 1: 334.

Hiney, M. and Olivier, G., 1999. Furunculosis (*Aeromonas salmonicida*). In Fish Diseases and Disorders, Volume 3, Viral Bacterial and Fungal Infections. (ed. Woo, P. T. K.and Bruno. D. W.). CABI publishing, New York., pp. 341-425.

Hispano, C., Nebra, Y. and Blanch, A. R.1997. Isolation in *Vibrio harveyi* from an ocular lesion in the short sunfish (*Mola mola*). *Bull. Eur. Assoc. Fish Pathol.*, 17, 3/4: 104-107.

Hjeltnes, B., Andersen, K. and Ellingsen, H-M., 1989. Vaccination against *Vibrio salmonicida*. The effect of different routes of administration and of revaccination. *Aquaculture.*, 83, 1-2: 1-6.

Hjeltnes, B. and Roberts, R. J., 1993. Vibriosis. In: Bacterial fish pathogens. (ed. Inglis, V. Roberts, R. J. and Bromage, N. R.). Blackwell Scientific Publications, Oxford, pp. 109-121.

Hoff, F. H., 1993. Marine ornamental fish culture. From discovery to commercialization (ed. Carrillo, M., Dahle, L., Morales, J., Sorgeloos, P., Svennevig, N., Wyban, J.). Oostende Belgium, *European Aaquaculture Soc.*, 19: 298.

Horne, M. T. and Barnes, A. C., 1999. Enteric redmouth disease (*Yersinia ruckeri*). In: Fish Diseases and Disorders, Volume 3, Viral Bacterial and Fungal Infections. (ed. Woo, P. T. K. and Bruno, D. W.), CABI publishing, New York., 455-477.

Horsely, R. W., 1973. The bacterial flora of Atlantic salmon (*Salmo salar* L.) in relation to its environment. *J. Appl. Bacteriol.*, 36, 377-386.

*Hoyt, R. E. and Levine, M. G., 1947. Method for determining sensitivity to penicillin and streptomycin. *Science*, 106: 171.

*Hrubec, T. C., Robertson, J. L., Smith, S. A. and Tinker, M. K., 1996. The effect of temperature and water quality on antibody response to *Aeromonas salmonicida* in sunshine bass (*Morone chrysops* x *Morone saxatilis*). *Vet. Immunol. Immunopathol.*, 50, 1-2: 157-166.

*Hucker, G. J. and Conn, H. J., 1923. Methods of Gram Staining. Tech. Bull. N. Y. Agric. Exp. Sta., 93.

Hugh, R. and Leifson, E., 1953. The taxonomic significance of fermentative versus oxidative metabolism of carbohydrates by various Gram-negative bacteria. *J. Bacteriol.*, 66: 24-26.

Iida, T., Sakata, C., Kawatsu, H. and Fukuda, Y., 1997. Atypical *Aeromonas salmonicida* infection in cultured marine fish. *Fish Pathol.*, 32: 65-66.

Iida, T., Wakabayahi, H. and Egusa, S., 1982. Vaccination for control of streptococcal disease in cultured yellowtail. *Fish Pathol.*, 16: 201-206.

Ilan, M., Contini, H., Carmeli, S. and Rinkevich, B., 1996. Progress towards cell cultures from a marine sponge that produces bioactive compounds. *J. Mar. Biotechnol.*, 4, 3: 145-149.

Inamura, H., Muroga, K. and Nakai, T. 1984. Toxicity of extracellular products of *Vibrio anguillarum*. *Fish Pathol.*, 19, 2: 89-96.

Inamura, H., Nakai, T. and Muroga, K., 1985. An extracellular protease produced by *Vibrio anguillarum*. *Bull. Jpn. Soc. Sci. Fish.*, 51, 12: 1915-1920.

Inglis, V. and Hendrie, M. S., 1993. *Pseudomonas* and *Alteromonas* infections. In: Bacterial fish pathogens (ed. Inglis, V. Roberts, R. J. and Bromage, N. R.). Blackwell scientific publications, London, pp. 169-174.

Ishida, N., 1992. Tissue levels of oxolinic acid after oral or intravascular administration to freshwater and sea water rainbow trout. *Aquaculture*, 102: 9-15.

Janssen, W. A. and Surgalla, M. J. 1968. Morphology, physiology and serology of a *Pasteurella* species pathogenic for white perch (*Roccus americanus*). *J. Bacteriol.*, 96: 1606-1610.

*Johnstone, J., 1927. Diseased conditions of fishes. *Proc. Trans. Liverpool Biol. Soc.*, 41: 162-167.

Joseph, J., Surendran, P. K. and Perigreen, P. A., 1988. Studies on the iced storage of cultured rohu (*Labeo rohita*). *Fish. Technol.*, 25: 105-109.

*Jung, J. H., Shin, J., Seo, Y., Sim and Chung J., 1995. Bioactive compounds from the marine sponges *Pachastrelia* sp. and *Jaspis* sp. In: Abstracts from the International Congress on Natural Products Research (ed. Chandler, R. F., Jurgens, T. M., Jurgens, A. R.). 14, 2: 138.

*Kaiser, H., Britz, P., Endemann, F., Haschick, R., Jones, C. L. W., Koranteng, B., Kruger, D. P., Lockyear, J. F., Oellermann, L. K., Olivier, A. P., Rouhani, Q. and Hecht, T., 1997. Development of technology for ornamental fish aquaculture in South Africa. *S. Afr. J. Sci.*, 93, 8: 351-354.

Kaper, J. B., Lockman, H. and Colwell, R. R., 1981. *Aeromonas hydrophila*: ecology and toxicology of isolates from an estuary. *J. Appl. Bacteriol.*, 50: 359-377.

Kawahara, E., Sako, H., Nomura, S. and Kusuda, R., 1989. Hemolysin production by beta-hemolytic *Streptococcus* sp. isolated from *Seriola quinqueradiata*. *Fish Pathol.*, 14, 219-223.

Kawakami, H., Shinohara, N., Fukuda, Y., Yamashita, H., Kihara, H., Sakai, M., 1997. The efficacy of lipopolysaccharide mixed chloroform-killed cell (LPS-CKC) bacterin of *Pasteurella piscicida* on Yellowtail, *Seriola quinqueradiata*. *Aquaculture.*, 154, 2: 95-104.

Kawano, K., Aoki, T. and Kitao, T., 1984. Duration of protection against vibriosis in ayu *Plecoglossus altivelis* vaccinated by immersion and oral administration with *Vibrio anguillarum*. *Nippon Suisan Gakkaishi*, 50: 771-774.

Kevin, H. A., (ed.), 1985. Procedures for the detection and identification of certain fish pathogens, 3rd edition, Fish Health Station, American Fisheries Society, Corvallis, Oregon, pp. 114.

Khalil, A.H. and Mansour, E. H., 1997. Toxicity of crude extracellular products of *Aeromonas hydrophila* in tilapia, *Tilapia nilotica*. *Lett. Appl. Microbiol.*, 25, 4, 269-273.

Kimura, H. and Kusuda, R., 1982. Studies on the pathogenesis of streptococcal infection in cultured yellowtails *Seriola* spp.: effect of crude exotoxin fractions from cell-free culture on experimental streptococcal infection. *J. Fish Dis.*, 5, 471-478.

Kinnunen, P. R., Bernardet, J. F. and Bloigu, A., 1997. Yellow pigmented filamentous bacteria connected with farmed salmonid fish mortality. *Aquaculture*, 149,1-2: 1-14.

*Klesius, P. H. and Shoemaker, C. A., 1999. Development and use of modified live *Edwardsiella iactaluri* vaccine against enteric septicaemia of catfish. In: Advances in Veterinary Medicine (ed. Schultz, R.). 41: 523-537.

Klesius, P. H., Shoemaker, C. A. and Evans, J. J., 2000. Efficacy of single and combined *Streptococcus iniae* isolate vaccine administered by intraperitoneal and intramuscular routes in tilapia (*Oreochromis niloticus*). *Aquaculture*, 188: 237-246.

*Kodama, H., Moustafa, M., Mikami, T. and Izawa, H., 1985. Characterisation of extracellular substances of *Vibrio anguillarum* toxic for rainbow trout and mice. *Microbiol. Immunol.*, 29: 909-920.

Kou, G. H., 1973. Studies on the fish pathogen, *Aeromonas liquefaciens*, II. The connections between pathogenic properties and the activities of toxic substances. *J. Fish. Soc. Taiwan*, 2: 42-46.

*Kovacs, N., 1928. Eine Vereinfachte methods zum machines der indole bildung durch bakterein. *Z. Immunforsch Exp. Ther.*, 55, 311.

*Kovacs, N., 1956. Identification of *Pseudomonas pyocyanea* by the oxidase reaction. *Nature*, London, 178, 703.

*Krantz, G. E., Redcliff, J. M. and Heist, C. E., 1963. Development of antibodies against *Aeromonas salmonicida* in trout. *J. Immunol.*, 91: 757-760.

Krantz, G. E., Redcliff, J. M. and Heist, C. E., 1964. Immune response of trout to *Aeromonas salmonicida:* Part I. Development of agglutinating antibodies and protective immunity. *Prog. Fish-Cult.*, 26,1: 3-10.

Kreger, A. S., 1984. Cytolytic activity and virulence in *Vibrio damsela*. *Infect. Immun.*, 44: 326-331.

Kreuter, M. H., Robitzki, A., Chang, S., Steffen, R., Michaelis, M., Kljajic, Z., Bachmann, M., Schroeder, H. C., Mueller, W. E. G., 1992. Production of the cytostatic agent aeroplysinin by the sponge *Verongia aerophoba* in *in vitro* culture. *Comp. Biochem. Physiol.*, C., 101C, 1:183-187.

Kumar, B. E., 1998. Domestic and export market potential of live aquarium fishes. Seminar on ornamental fishery. All India aquarium show and exhibition, 1998, Trivandrum, India, 5pp.

*Kuo, Tzong Fu and Chung, Cheng Dar, 1994. Drug resistance studies of pathogenic bacteria isolated from aquarium fishes against up to 8 antimicrobics. *Mem. Coll. Agric. Natl. Taiwan Univ.*, 34, 3: 247-260.

Kusuda, R. and Hamaguchi, M., 1988. Extracellular and intracellular toxins of *Streptococcus* sp. isolated from yellowtail. *Bull. Eur. Assoc. Fish Pathol.*, 8: 9-10.

Kusuda, R. and Kimura, H., 1978. Studies on the pathogenesis of streptococcal infection in cultured yellowtails, *Seriola*, spp: the fate of *Streptococcus* sp. bacteria after inoculation. *J. Fish Dis.*, 1: 109- 114.

Kusuda, R., Sako, S. and Kawai, K., 1979. Classification of vibrios isolated from diseased fishes-I. On the morphological, biological and biochemical properties. *Fish Pathol.*, 13: 123-137.

Kusuda, R. and Taki, H., 1973. Studies on a nocardial infection of cultured yellowtail. I. On the morphological, biological characteristics of *Nocardia* isolated from diseases fishes. *Bull. Jpn. Soc. Sci. Fish.*, 39: 937-943.

Laemmli, U. K., 1970. Cleavage of structural proteins during assembly of the head of bacteriophage T4. *Nature*, London, 227: 680-685.

Lakshmanan, M., Jayaraman, K., Jayaraman, J. and Ganesan, A., 1971. Laboratory experiments in microbiology and molecular biology. Higginbotham's Ltd., India. 115pp.

Lang, T., Peters, G., Hoffmann, R. and Meyer, E., 1987. Experimental investigations on the toxicity of ammonia: effects on ventilation frequency, epidermal mucous cells, and gill structure of rainbow trout, *Salmo gairdneri*. *Dis. Aquat. Org.*, 3: 159-165.

Laurent, P., Maina, J. N., Bergman, H. L., Narahara, A., Walsh, P. J. and Wood, C. M., 1995. Gill structure of a fish farm from an alkaline lake: effect of short term exposure to neutral conditions. *Can. J. Zool.*, 73: 1170-1181.

Lee, K. K., 1995. Pathogenesis studies on *Vibrio alginolyticus* in the grouper, *Epinephelus malabaricus*, Bloch et Schneider. *Microb. Pathog.*, 19, 1: 39-48.

*Len, P. P., 1987. Mesophilic spoilage of marine fish: Bay trout (*Arripis trutta*), bream (*Acanthopagrus butcheri*) and mullet (*Aldrichetta forsteri*). *Food Technol. Aust.*, 39, 6: 277-282.

Leong, T. S., 1993. Chemotherapy in aquaculture. In: Diseases in aquaculture: the current issues. (ed. Subhasinge, R. P. and Shariff, M.). Malaysian Fisheries Society, Selangor Darul Ehsan, Malaysia, pp. 125-136.

Lewis, W. M. and Morris, D. P., 1986. Toxicity of nitrite to fish: a review. *Trans. Am. Fish. Soc.*, 115: 183-195.

Lightner, D. V., Redman, R., Mohney, L., Dickenson, G. and Fitzsimmons, K., 1988. Major diseases encountered in controlled environment culture of tilapias in fresh and brackish water over a three year period in Arizona. In: The second international symposium on tilapia in aquaculture. (ed. Pullin, R. S. V., Bhukaswan, T., Tonguthai, K., Maclean, J. L.). Bangkok Thailand, 15: 111-116.

*Lillie, R. D., 1928. The Gram stain. A quick method for staining Gram-positive organisms in the tissues. *Arch. Path.*, 5: 828.

*Lin, C. L., Chen, S. N., Kou, K. H., Wu, C. L. and Ting, Y. Y., 1993. The diseases of cultured grey mullet (*Mugil cephalus* Linnaeus) during the culture. *Coa fish. Ser.*, 40: 47-60.

Lipton, A. P., 1987. Studies on the microbial diseases of some commercially important fresh water fishes with special reference to *Aeromonas* and *Pseudomonas* spp. Ph. D. thesis submitted to the Madurai Kamaraj University. 244pp.

Lipton, A. P., 1993. *Cryptocaryon irritans* (Protozoa: Ciliata) infection among aquarium held marine ornamental fish and its control. *Curr. Sci.*, 65, 7: 571- 572.

Lipton, A. P., 2001. Final report of the ICAR Ad-hoc project, "Studies on the disease management in fish/shellfish farming using bioactive substances from marine organisms". Submitted to the ICAR. 40p.p.

Liston, J., 1957. The occurrence and distribution of bacterial types on flatfish. *J. Gen. Microbiol.*, 16: 205-216.

Liu, P. C., Lee, K. K. and Chen, S. N., 1996. Pathogenicity of different isolates of *Vibrio harveyi* in tiger prawn, *Penaeus monodon*. *Lett. Appl. Microbiol.*, 22: 413-416.

Liu, P. V., 1957. Survey of hemolysin production among species of *Pseudomonas*. *J. Bacteriol.*, 74: 718-727.

Llewellyn, L. C., 1980. A bacterium with similarities to the red mouth bacterium and *Serratia liquefaciens* (Grimes and Hennerty) causing mortalities in hatcheryreared salmonids in Australia. *J. Fish Dis.*, 3, 1: 29-39.

Lockyear, J., Kaiser, H. and Hecht, T. Studies on the captive breeding of the Knysna seahorse, *Hippocampus capensis*. *Aquarium Sci. Conserv.*,1, 2: 129-136.

Love, M., Teebeken-Fisher, D., Hose, J. E. Farmer, J. J. III, Hickman, F. W. and Fanning, G. R., 1981. *Vibrio damsela*, a marine bacterium, causes skin ulcers on the damselfish, *Chromis punctipinnis*. *Science*, 214: 1139-1140.

Lowrie, J. and Borneman, E., A., 2000. Survey of the Marine Microbes. Aquarium frontiers homepage: http://www.animalnetwork.com/fish2/aqfm/default.asp.

Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J., 1951. Protein measurement with the folin phenol reagent. *J. Biol. Chem.*, 193: 265-275.

*Lustigman, B., Lee, L. H., Thees, N. and Masucci, J., 1992. Production of antibacterial substances by macroalgae of the New York/New Jersey coast, USA. *Bull. Environ. Contam. Toxicol.* , 49, 5: 743-749.

*Mackie, T. J. and McCartney, 1953. Handbook of bacteriology, 9th edition. Edinburgh, Livingstone.

*Magalhaes, V., Castello Filho, A., Magalhaes, M. and Gomes, T. T., 1993. Laboratory evaluation on pathogenic potentialities of *Vibrio furnissii*. Mem. Inst. Oswaldo Cruz, 88, 4: 593-597.

Magariños, B., Santos, Y., Romalde, J. L., Rivas, C., Barja, J. L. and Toranzo, A. E., 1992. Pathogenic activities of live cells and extracellular products of the fish pathogen, *Pasteurella piscicida*. *J. Gen. Microbiol.*, 138: 2491-2498.

Mary, A., Mary, V., Sarojini, R. and Nagabhushanam, R., 1994. Bacteriostatic compounds in extracts of marine animals from the Indian Ocean. In: Recent developments in biofouling control (ed. Thompson, M.F., Nagabhushanam, R., Sarojini, R., Fingerman, M.) New Delhi, India. Oxford and IBH, pp. 229-239.

Maule, A. G., Tripp, R. A., Kaattari, S. L. and Schreck, C. B., 1989. Stress alters immune function and immune fractions and disease resistance in channel Chinook salmon (*Oncorhynchus tshawytscha*). *J. Endocri.*, 120:135-142.

Mazzolini, E., Fabris, A., Ceschia, G., Vismara, D., Magni, A., Amadei, A., Passera, A., Danielis, L. and Giorgetti, G., 1998. Pathogenic variability of *Pasteurella piscicida* during *in vitro* cultivation as a preliminary study for vaccine production. *J. Appl. Ichthyol.*, 14, 3-4: 265-268.

McCarthy, D. H., Croy, T. R. and Amend, D. F., 1984. Immunisation of rainbow trout *Salmo gairdneri* Richardson, against bacterial kidney disease: preliminary efficacy evaluation. *J. Fish Dis.*, 7: 65-71.

McCarthy, D. H., Stevenson, J. P. and Salsbury, A. W., 1974. Combined *in vitro* activity of trimethoprim and sulphonamides on fish pathogenic bacteria. *Aquaculture*, 3: 87-91.

McIntosh, D. and Austin, B., 1990. Recovery of an extremely proteolytic form of *Serratia liquefaciens* as a pathogen of Atlantic salmon, *Salmo salar* in Scotland. *J. Fish Biol.*, 36: 765-772.

Meade, J. W., 1985. Allowable ammonia for fish culture. *Prog. Fish-Cult.*, 47: 135-145.

Mellergaard, S. and Dalsgaard, I., 1987. Disease problems in Danish eel Farms. *Aquaculture*, 67: 139-146.

*Michael, C., Gerard, J. P., Fourbet, B., Collas, R. and Chevalier, R., 1980. Emploi de la flumequine contre la furunculose des salmonides: essais therapeutiques et perspectives pratiques. *Bull. Francais Piscic.*, 52: 154-162.

Midtlyng, P. J., Reitan, L. J. and Speilberg, L., 1996. Experimental studies on the efficacy and side effects of intraperitoneal vaccination of Atlantic salmon (*Salmo salar* L.) against furunculosis. *Fish Shellfish Immunol.*, 6, 5: 335-350.

*Miller, L. C. and Trainter, M. L., 1944. *Proc. Soc. Exptl. Biol. Med.*, 57, 261.

*Mohs, F. S., 1945. A simple quantitative test for the penicillin sensitivity of bacteria: the 'radial streak' method. *J. Lab. clin. Med.*, 30: 800-802.

Mualu, F. S. and Ijumba, P. K., 1982. Importance of the marine environment and marine fish in human microbial diseases. Univ. Sci. J., Dar-Es-Salaam, 8, 1-2: 89-100.

Mudarris, M., Austin, B., Segers, P., Vancanneyt, M., Hoste, B. and Bernardet, J. F., 1994. *Flavobacterium scophthalmum* sp. nov., a pathogen of turbot (*Scophthalmus maximus* L.). *Int. J. Syst. Bacteriol.*, 44, 3: 447-453

Munro, A. L. S., 1982. The pathogenesis of bacterial diseases of fishes. In: Microbial disease of fish (ed. Roberts, R. J.), Acedemic press, London, pp. 131-149.

Munro, P. D., Barbour, A. and Birbeck, T. H., 1994. Comparison of the gut bacterial flora of start-feeding larval turbot reared under different feeding conditions *J. Appl. Bacteriol.*, 77: 560-566.

Muroga, K and Egusa, S., 1967. *Vibrio anguillarum* from an endemic disease of ayu in Lake Hamana. *Bull. Jpn. Soc. Sci. Fish.*, 33: 636-640.

Muroga, K. Higashi, M. and Keitoku, H., 1987. The isolation of intestinal microflora of farmed red sea bream (*Pagrus major*) and black sea bream

(*Acanthopagrus schlegeli*) at larval and juvenile stages. *Aquaculture*, 65: 79-88.

Nelson, E. J. and Ghiorse, W. C., 1999. Isolation and identification of *Pseudoalteromonas piscicida* strain Cura-d associated with diseased damsel fish (Pomacentridae) eggs. *J. Fish Dis.*, 22: 253-260.

Newman, J. T., Cosenza, B. J. and Buck, J. D., 1972. Aerobic microflora of the bluefish intestine. *J. Fish Res. Bd. Canada*, 29: 333-336.

Newman, S. G., 1993. Bacterial vaccines for fish. *Annu. Rev. Fish Dis.*, 3: 145-185.

Newton, J. C., Wolfe, L. G., Grizzle, J. M. and Plumb, J. A., 1989. Pathology of experimental enteric septicaemia in channel catfish, *Ictalurus punctatus* (Rafinesque), following immersion exposure to *Edwardsiella ictaluri*. *J. Fish Dis.*, 12: 335-347.

Nieto, T. P., López, L. R., Santos, Y., Núñez, S. and Toranzo, A. E., 1990. Isolation of *Serratia plymuthica* as an opportunistic pathogen in rainbow trout, *Salmo gairdneri* Richardson. *J. Fish Dis.*, 13: 175-177.

Nigrelli, R. F. and Ruggieri, G. D., 1965. Studies on the viral diseases of fishes. Spontaneous and experimentally induced cellular hypertrophy (Lymphocytes disease) in fishes in the New York aquarium, with a report on new cases and an annotated bibliography (1874-1965). *Zoologica*. (N.Y.), 50: 83-96.

Noble, A. C., 1996. Major diseases encountered in rainbow trout reared in recirculating systems. Aquacultural Engineering Society Proceedings II: Successes And Failures In Commercial Recirculating Aquaculture, 1: 17-27.

Noble, A. C. and Summerfelt, S. T., 1996. Diseases encountered in rainbow trout cultured in recirculating systems. *Annu. Rev. Fish Dis.*, 6: 65-92.

*Nogi, Y., Nakase, T. and Horikoshi, K., 1997. Taxonomic studies about new species of the genus *Flavobacterium* isolated from Japan Trench. Rep. Japan Mar. Sci. Technol. Cent., Kaiyo Kagaku Gijutsu Senta Shiken Kenkyu Hokoku, 35: 77-84.

Norqvist, A., Normann, B. and Wolf-Watz, H., 1990. Identification and characterisation of a zinc metalloprotease associated with invasion by the fish pathogen *Vibrio anguillarum*. *Infect. Immun.*, 58: 3731-3736.

Novotny, A. J., 1978. Vibriosis and furunculosis in marine cultured salmon in Puget Sound, Washington, *Mar. Fish. Rev.*, 40, 52-55.

*Oestmann, D. J., 1987. Disease in the ornamental marine aquarium. *Vet. Annu.*, 27: 361-366.

*Olivier, G., Moore, A. R. and Fields, J., 1990. Taxonomy and virulence of atypical strains of *A.salmonicida* isolated from salmonid and non-salmonid fish. Abstract. International Conference on Bacterial Diseases of Fish. Stirling, Scotland.

Oppenheimer, C. H. and Kesteven, G. L., 1953. Disease as a factor in natural mortality of fish. FAO Fish. Bull., 6: 215-222.

*Ortega, C., Muzquiz, J. L., Fernandez, A., Ruiz, I., De Blas, I., Simon, M. C. and Alonso, J. L., 1996. Water quality parameters associated with *Aeromonas* spp. affected hatcheries. *Vet. Res.*, 27, 6: 553-560.

Ortigosa, M., Garay, E. and Pujalte, M. J., 1995. Numerical taxonomy of aerobic, Gram-negative bacteria associated oysters and surrounding seawater of the Mediterranean coast. *Syst. Appl. Microbiol.*, 17, 4: 589-600.

Ounaies, N., 1998. Ornamental fish and their commercial potential. In: Tropical and island aquaculture. Aquaculture Insulaire Et Tropicale. (ed. Lacroix, D. Fuchs, J.), Plouzane, France IFREMER, 20: 99-100.

Pacha, R. E. and Ordal, E. J., 1970. Myxobacterial diseases of salmonids. In: A Symposium on Diseases of Fishes and Shellfishes (ed. S. F. Sniezko). American Fisheries Society, Washington DC., Special publication no. 5: 243-257.

Paperna, I., 1980. *Amyloodinium ocellatum* (Brown, 1931) (Dinoflgellida) infestations in cultured marine fish at Eliat, Red Sea: Epizootiology and Pathology. *J. Fish Dis.*, 3: 363-372.

Papoutsoglou, S., Costello, M. J., Stamou, E. and Tziha, G., 1996. Environmental conditions at sea cages, and ectoparasites on farmed European sea bass, *Dicentrarchus labrax* (L.), and gilt head sea bream, *Sparus aurata* L., at two farms in Greece. *Aquacult. Res.*, 27, 1: 25-34.

Paterson, W. D., Desautels, D. and Weber, J. M., 1981. The immune response of Atlantic salmon, *Salmo salar* L., to the causative agent of bacterial kidney disease, *Renibacterium salmoninarum*. *J. Fish Dis.*, 4: 99-111.

Paterson, W. D. and Fryer, J. L., 1974 a. Immune response of juvenile Coho salmon (*Oncorhynchus kisutch)* to *Aeromonas salmonicida* cells administered intraperitoneally in Freund's complete adjuvant. *J. Fish Res. Bd. Canada.*, 31,11: 1751-1755.

Paterson, W. D. and Fryer, J. L., 1974 b. Effect of temperature and antigen dose on antibody response of juvenile Coho salmon (*Oncorhynchus kisutch)* to

Aeromonas salmonicida endotoxin. *J. Fish Res. Bd. Canada.*, 31: 1743-1749.

Pfister, R. M. and Burkholder, P. R., 1965. Numerical taxonomy of some bacteria isolated from Antarctic and tropical seawater. *J. Bacteriol.*, 90: 863-872.

*Pichai Sonchaeng, 1988. Taxonomic study on butterfly fish in Thai waters. Abstracts of Master of Science Theses Fisheries Science, 13: 8.

Pickering, A. D. and Duston, J., 1983. Administration of cortisol to brown trout, *Salmo trutta*, and effects on the susceptibility to *Saprolegnia* infection and furunculosis. *J. Fish Biol.*, 21: 163-175.

Piddock, L J. V., 1990. A review. Techniques used for the determination of antimicrobial resistance and sensitivity in bacteria. *J. Appl. Bacteriol.*, 68. 307-318.

Plumb, J. A. 1992. Disease control in aquaculture. In: Diseases in Asian aquaculture. 1. Proceedings of the first symposium on diseases in Asian aquaculture, (ed. Shariff, M., Subasinghe, R. P., Arthur, J. R.). Bali, Indonesia. Manila-Philippines fish health section, Asian Fisheries Society. pp. 3-17.

Plumb, J. A., 1999. *Edwardsiella* septicaemia. In: Fish Diseases and Disorders volume 3, Viral Bacterial and Fungal Infections. (ed. Woo, P. T. K. and Bruno. D. W.), CABI publishing, New York., pp. 479-521.

Plumb, J. A. and Sanchez, D. J., 1983. Susceptibility of five species of fish to *Edwardsiella ictaluri*. *J. Fish Dis.*, 6: 261-266.

*Pollack, M. R., 1962. In "The bacteria" (ed. Gensalua and Stanier, R. Y.) Academics Press, New York., 4, pp. 121-175.

*Porcile, P., Bandelloni, R., Tiberti, D. and Mandolini, F., 1991. Biologically active compounds in some algae and their interactions with marine bacteria: The ecological role. *Oebalia*, 17, no. Suppl. 2: 455-465.

*Prado, B., Llanos, J., del Moral, A., Cid, E., Magana, E., Diaz, R. and Garcia Tello, P., 1992. Numerical taxonomy and habitat of marine bacteria isolated from Montemar, 5 region, Valparaiso, Chile. *Rev. Biol. Mar.*, 27, 1: 37-42.

Price, N. C., Stevens, L., Duncan D. and Snodgrass, M., 1989. Proteases secreted by strains of *Aeromonas salmonicida*. *J. Fish Dis.*, 12: 223-232.

*Pullium, J. K., Dillehay, D. L. and Webb, S., 1999. High Mortality in Zebra fish (*Danio rerio*) *Contemp. Topics Lab. Anim. Sci.*, 38, 3: 80-83.

*Qin, Q. and Pan, J., 1996. Safety and efficacy of *Vibrio vulnificus* bacterins vaccination by injection administration against vibriosis in *Epinephelus awoara*. Trop. *Oceanol. Redai haiyang*, 15, 2: 7-12.

Quentel, C. and Ogier de Baulny, M., 1995. Vaccination of juvenile turbot, *Scophthalmus maximus* L., against vibriosis. *Aquaculture*, 132: 125-131.

Rao, D. S., Girijavallabhan, K. G., Muthusamy, S., Chandrika, V., Gopinathan, C. P., Kalimuthu, S. and Najmuddin, M., 1991. Bioactivity in marine algae. In: Bioactive compounds from marine organisms with emphasis on the Indian Ocean. An Indo United States symposium. (ed. Thompson, M. F., Sarojini, R., Nagabhushanam, R.) pp. 373-377.

*Ratnasooriya, W. D., Premakumara, G. A. S., Tillekeratne, L. M. V., 1996. Gastroprotective activity of crude extract of *Jania* sp. (red algae) on ethanol-induced gastric lesions in rats. *Med. Sci. Res.*,18, 20: 803-804.

*Reddish, G. F., 1929. Methods of testing antiseptics. *J. Lab. clin. Med.*, 14: 649-658.

Reddy, P. K. and Leatherland, J. F., 1998. Stress physiology. In: Fish diseases and disorders. Volume 2. Non infectious disorders (ed. Leatherland, J. F. and Woo, P. T. K.), CABI publishing, New York, 279-301.

*Reed, L. J. and Muench, H., 1938. A simple method of estimating fifty percent end points. *Am. J. Hygiene*, 27: 493-497.

Riddle, L. M., Graham, R. L. Amborski and Hugh-Johnes, M., 1981. Production of hemolysin and protease by *Aeromonas hydrophila* in a chemically defined medium. *Develop. Biol. Standard.*, 49: 125-133.

Rigos, G., Grigorakis, K., Nengas, I., Christophilogiannis, P., Yiagnisi, M., Koutsodimou, M., Andriopoulou, A. and Alexis, M., 1998. Stress related pathology seems a significant obstacle for the intensive farming of common dentex, *Dentex dentex* (Linnaeus 1758). *Bull. Eur. Assoc. Fish Pathol.*,18, 1: 15-18.

Ringø, E., Strom, E. and Tabachek, J. A., 1995. Intestinal microflora of salmonids- a review. *Aquacult. Res.*, 26, 773-789.

Roberts, R. J., 1989. Fish pathology. 2nd edition, Balliere, Tindall, London, 452 pp.

Rodriguez, L. A., Ellis, A. E. and Nieto, T. P., 1992. Purification and characterisaton of an extracellular metalloprotease, serine protease and hemolysin of *Aeromonas hydrophila* B_{32} strains. *J. Fish Dis.*, 16: 73-78.

Rodriguez, L. A., Gallardo, C. S., Acosta, F., Nieto, T. P., Acosta, B. and Real, F., 1998. *Hafnia alvei* as an opportunistic pathogen causing mortality in brown trout, *Salmo trutta* L. *J. Fish Dis.*, 21: 365-370.

Rogers, W. A., Xu, D., 1992. Protective immunity induced by a commercial Vibrio vaccine in hybrid striped bass. *J. Aquat. Anim. Health*, 4, 4: 303-305.

Rohovec, J. S., Winton, J. R. and Fryer, J. L., 1981. Bacterins and vaccines for control of infectious diseases fish. In: Proceedings of Republic of China-United States Cooperative Science Seminar on Fish Diseases (NSC Symposium Series no. 3). National Science Council, Republic of China. 115-121.

*Ross, A. J., 1960. *Mycobacterium salmoniphilum* sp. nov. from salmonid fishes. *Am. Rev. Respir. Dis.*, 81: 241-250.

*Ross, A. J. Earp, B. J. and Wood, J. W., 1959. Mycobacterial infections in adult salmon and steel head trout returning to the Columbia River basin and other areas in 1957. U. S. Fish Wildl. Serv., Spec. Sci. Rep.-fish, 332, 1-34.

Ross, A. J. and Klontz, G. W., 1965. Oral immunisation of rainbow trout (*Salmo gairdneri*) against an etiologic agent of "redmouth disease". *J. Fish Res. Bd. Canada*, 22: 713-719.

Rucker, R. R., Burnier, A. F., Whipple, W. H. and Burrows, R., 1951. Sulfadiazine for kidney disease. *Prog. Fish-Cult.*, 13:135-137.

Saeed, M. O., 1995. Association of *Vibrio harveyi* with mortalities in cultured marine fish in Kuwait. *Aquaculture*, 136, 1-2: 21-29.

Sakai, M., Aoki, T., Kitao, T., Rohovec, J. S. and Fryer, J. L. 1986. Fluctuation in the number of bacterial cells in organs of vaccinated fish after artificial challenge. *Bull. Jpn. Soc. Sci. Fish.*, 52, 2: 249-255.

*Sakata, T., Okabayashi, J. and Kakimoto, D., 1978. Microflora in the digestive tract of marine fish II. Mem. Fac. Fish. Kogoshima Univ., 27, 73-78.

Sakata, T., Okabayashi, J. and Kakimoto, D., 1980. Variations in the intestinal microflora of *Tilapia* reared in and fresh and seawater. *Bull. Jpn. Soc. Sci. Fish.*, 46: 967-975.

Sakazaki, R, 1974. Genus *Serratia*. In: Bergy's manual of determinative bacteriology (ed. Buchanan, R. E. and Gibbons, N. E.). 8th edition. Williams and Wilkins Company, Baltimore, pp. 326.

*Sakazaki, R. and Tamura, K. 1992. The genus *Hafnia*. In: The Prokaryotes, Vol. III, 2nd edn. (ed. Ballows, A., Trüper, H. G., Dworkin, M., Harder, W. and Schleifer, K.). Springer-Verlag, Berlin, pp. 2816-2821.

Sanders, J. E. and Fryer, J. L., 1988. Bacteria of Fish. In: Methods in Aquatic Bacteriology, (ed. Austin, B.), John Wiley and Sons Ltd. Chichester, pp. 115-142.

Santos, Y., Bandin, I., Nuñez, S., Montero, M., Silva, A. and Toranzo, A.E., 1992. Comparison of the extracellular biological activities of *Vibrio anguillarum* and *Aeromonas hydrophila*. *Aquaculture*, 107, 2-3: 259-269.

Santos, Y., Bandin, I., Toranzo, A. E., 1996. Immunological analysis of extracellular products and cell surface components of motile *Aeromonas* isolated from fish. *J. Appl. Bacteriol.*, 81, 6: 585-593 .

Santos, Y., Lallier, R., Bandin, I., Lamas, J. and Toranzo, A. E., 1991. Susceptibility of turbot (*Scophthalmus maximus)*, coho salmon (*Oncorhynchus kisutch*), and rainbow trout (*O. mykiss*) to strains of *Vibrio anguillarum* and their exotoxins. *J. Appl. Ichthyol.*, 7, 3: 160-167.

Schachte Jr., J. H, 1978. Immunisation of channel catfish, *Ictalurus punctatus*, against two bacterial diseases. *Mar. Fish Rev.*, 40, 3: 18-19.

Schachte Jr., J. H. and Mora, E. C., 1973. Production of agglutinating antibodies in the channel catfish (*Ictalurus puctatus*) against *Chondrococcus columnaris*. *J. Fish Res. Bd. Canada*, 30,1: 116-118.

*Schmith, K. and Reymann, F. E., 1940. Experimentelle og Kliniske undersogelser over gonococcers folsomhed over for sulfapyridine. *Nordisk Medicin*, 8: 2493-2499.

Schnick, R. A., 1988. The impetus to register new therapeutants for aquaculture. *Prog. Fish- Cult.*, 50, 190-196.

Schnick, R. A., 2001. International harmonization of antimicrobial sensitivity determination for aquaculture drugs. *Aquaculture*, 196, 3-4: 277-288.

Schreck, C. B., Contreras-Sanchez, W. and Fitzpatrick, M. S., 2001. Effects of stress on fish reproduction, gamete quality, and progeny. *Aquaculture*, 197, 1-4: 3-24.

*Shewan, J. M., 1961. The microbiology of seawater fish. In: Fish as Food, vol. 1. (ed. Borgstrom, G.). Academic Press, New York, pp. 487-560.

Shewan, J. M., Hobbs, G. and Hodgkiss, W., 1960. A determinative scheme for the identification of certain genera of Gram-negative bacteria with special reference to the Pseudomonadaceae. *J. Appl. Bacteriol.*, 23, 3: 379-390.

Shewan, J. M., Hodgkiss, W. and Liston, J., 1954. A method for rapid differentiation of certain non-pathogenic, asporogenous bacilli. *Nature*, 173: 208-209.

Shiraki, K., Miyamoto, F., Sato, T., Sonezaki, I. and Sano, K., 1970. Studies on a new chemotherapeutic agent nifurprazine (HB-115) against infectious diseases. Part I. *Fish Pathol.*, 4: 130-137.

Shoemaker, C. A., Klesius, P. H. and Bricker, J. M., 1999. Efficacy of a modified live *Edwardsiella ictaluri* vaccine in channel catfish as young as s seven days post hatch. *Aquaculture*, 176: 189-193.

Shome, R., Shome, B. R., Mandal, A. B. and Bandopadhyay, A. K., 1995. Bacterial flora in mangroves of Andaman. Part 1: Isolation, identification and antibiogram studies. *Indian J. Mar. Sci.*, 24: 97-98.

Shotts, E. B. and Bullock, G. L., 1975. Bacterial diseases of fishes; diagnostic procedures for Gram negative pathogens. *J. Fish Res. Bd. Canada*,, 32: 1243-1247.

Shotts, E. B., Kleckner, A. L., Gratzek, J. B. and Blue, J. L., 1976. Bacterial flora of aquarium fishes and their shipping waters imported from Southeast Asia. *J. Fish. Res. Bd. Canada*, 33: 732-735.

Shotts, E. B. Jr., Talkington, F. D., Elliot, D. G. and McCarthy, D. H., 1980. Aetiology of an ulcerative disease in goldfish, *Carassius oratus* L., characterisation of the causative agent. *J. Fish Dis.*, 3: 181-186.

Simidu, U. and Aiso, K., 1962. Occurrence and distribution of heterotrophic bacteria in sea water from Kamogawa bay. *Bull. Jpn. Soc. Sci. Fish.*, 28, 11: 1133-1141.

Simidu, U. and Kaneko, E., 1969. Microflora of fresh and stored flatfish, *Kareius bicoloratus*. *Bull. Jpn. Soc. Sci. Fish.*, 35. 77-82.

*Sin, T. M., Teo, M. M., Ng, P. K. L., Chou, L. M. and Khoo, H. W., 1994. The damselfishes (Pisces: Osteichthyes: Pomacentridae) of Peninsular Malaysia and Singapore: Systematics, ecology and conservation. In: Ecology and conservation of South East Asian marine and freshwater environments including wetlands. (ed. Sasekumar, A., Marshall. N., Macintosh, D. J.), 285, 1-3: 49-58.

Sindermann, C. J., 1990. Principal diseases of marine fish and shellfish. 2nd edition Volume 1. Diseases of marine fish. Academic Press, 521 pp.

Smart, G, 1976. The effect of ammonia exposure of gill structure of the rainbow trout (*Salmo gairdneri*). *J. Fish Biol.*, 8: 471- 475.

*Smith, P. E. and Piper, R. G., 1975. Lesions associated with chronic exposure to ammonia. In: The Pathology of Fishes (ed. Ribelin, W. E. and Migaki, G.). University of Wisconsin Press, Madison, Wisconsin, pp. 497-517.

Sniezko, S. F., 1978. Control of fish diseases. *Mar. Fish. Rev.*, 40, 3, 65-68.

*Sniezko, S. F. and Griffin, P.J., 1951. Successful treatment of ulcer disease in brook trout with Terramycin. *Science*, 112: 717-718.

Spanggaard, B., Huber, I., Nielsen, J., Nielsen, J., Appel, K. F. and Gram, L., 2000. The microflora of rainbow trout intestine: a comparison of traditional and molecular identification. *Aquaculture*, 182: 1-15.

*Speare, D. J., 1991. Endothelial lesions associated with gas bubble disease in fish. *J. Comp. Pathol.*, 104: 327- 335.

Speare, D. J., 1998. Non infectious diseases associated with intensive aquaculture industry. In: Fish diseases and disorders. Volume 2. Non infectious disorders (ed. Leatherland, J. F. and Woo, P. T. K.). CABI publishing, New York, pp. 303-333.

Stevenson, R. M. W. and Allan, B. J., 1981. Extracellular virulence products in *Aeromonas hydrophila*. Disease processes in salmonids. *Develop. Biol. Standard.*, 49: 173-180.

Stoskopf, M. K., 1993. Fish Medicine. W. B. Saunders Company, Philadelphia. 882 pp.

Strickland, J. D. H. and Parsons, T. R., 1968. A practical handbook of seawater analyses. *Bull. Fish. Res. Bd. Canada*, 167: 1-311.

Subasinghe, R. P., 1992. The use of chemotherapeutic agents in aquaculture in Sri Lanka. In: Diseases in Asian Aquaculture.1. Proceedings of the first symposium on diseases in Asian aquaculture. (ed. Shariff, M., Subasinghe, R. P., Arthur, J. R.). Manila Philippines fish health section, Asian Fisheries Society, pp. 547-553.

Sugahara, I., Kimura, T. and Hayashi, K., 1988. Distribution and generic composition of denitrifying bacteria in coastal and oceanic bottom sediments. *Bull. Jpn. Soc. Sci. Fish.*, 54, 6: 1005-1010.

Sugita, H., Noguchi, T., Hwang, D., Furuta, M., motokane, T., Sonoda, T., Hashimoto, K. and Deguchi, Y., 1987. Intestinal microflora of coastal pufferfishes. *Nippon Suisan Gakkaishi*, 53, 2201-2207.

Sugita, H. Shibuya, K., Hanada, H. and Deguchi, Y., 1987. Antibacterial abilities of intestinal microflora of the river fish. *Fish. Sci.*, 63: 378-383.

Sumpter, J. P., 1993. The deleterious effects of stress and their significance to aquaculture. Production, environment and quality, European aquaculture society, 18, 157-166.

*Supamataya, M., 1988. The study of diseases in sand goby (*Oxyeleotris marmoratus* Bleeker) in cage culture and some environmental factors

related to infection. Abstracts of Master of Science Theses, Fisheries Science, 13: 3.

Suprapto, H., Nakai, T. and Muroga, K., 1995. Toxicity of extracellular products and intracellular components of *Edwardsiella tarda* in the Japanese eel and flounder. *J. Aquat. Anim. Health*, 7, 292-297.

Surendran, P. K., 1980. Chemical preservatives in relation to control of microbial changes in fishery products. Ph. D. Thesis, University of Kerala, Trivandrum.

Surendran, P. K. and Gopakumar, K., 1981. Selection of bacterial flora in the chlortetracycline treated oil sardine (*Sardinella longiceps*), Indian mackerel and prawn (*Metapenaeus dobsoni*) during ice storage. *Fish. Technol.*, 18: 133-141.

*Sutherland, P. L., 1922. A tuberculosis like disease in salt water fish (Halibut) associated with the presence of an acid fast tubercle like bacillus. *J. Pathol. Bacteriol.*, 25, 31-35.

*Tajima, K., Takahashi, T., Ezura, Y and Kimura, T., 1983. Studies on the virulence factors produced by *Aeromonas salmonicida*, causative agent of furunculosis in salmonids. II. Studies on the pathogenicity of the protease of *Aeromonas salmonicida* artificial infection-4 (EFDL) on yamabe (*Oncorhynchus masou* f. *ishikawai*) and goldfish (*Carassius auratus*) and the substance which exhibits cytotoxic effects on RTG-2 (Rainbow trout gonad) cells. *Buil. Fac. Fish. Hokkaido Univ.*, 34, 111-123.

Tanasomwang, V. and Muroga, K., 1990. Intestinal microflora of marine fishes at their larval and juvenile stages. In: Proceedings of the second Asian fisheries forum (ed. Hirano, R., Hanyu, I.). Asian Fisheries Society. pp. 647-650,

Tebbit, G. L., Erickson, J. D. and Vande Water, 1981. Development and use of *Yersinia ruckeri* bacterins to control enteric redmouth disease. *Develop. Biol. Standard.*, 49: 395-401.

Tebbit, G. L. and Goodrich, T. D., 1983. Vibriosis and the development of effective bacterins for its control. In "Antigens of Fish Pathogens" (ed. Anderson,D. P., Dorson, M. and Dubourget, P.), Merieux, Lyon, France, pp. 225-248.

Thampuran, N. and Surendran, P. K., 1998. Incidence of motile aeromonads in marine environment, fishes and processed fishery products. In: Technological advancements in fisheries, Proceedings of the National Symposium on Technological Advancements in Fisheries and its Impact on Rural Development. (ed. Hameed, M. S., Kurup, B. M.), Cochin-India. pp. 352-358.

*Thornsberry, C., Gavan, T. L. and Gerlach, E. H., 1977. New developments in antimicrobial agent susceptibility testing. In Cumitech 6 (ed. Sherris, J. C.), Washington: American Society for Microbiology.

*Thornton, J. C., 1995. Furunculosis vaccines: The next generation. Proceedings of the second BCMAFF on furunculosis. Managing furunculosis in the '90S. no. 95-3 pp. 38-41.

Thune, R. L., Collins, L. A. and Pena, M. A., 1997. A comparison of immersion, immersion / oral combination and injection vaccination of channel catfish *Ictalurus punctatus* against *Edwardsiella ictaluri*. *J. World Aquacult. Soc*. 28: 193-201.

Thune, R. L., Graham, T. E., Riddle, L. M. and Amborski, R. L., 1982. Extracellular proteases from *Aeromonas hydrophila*: partial purification and effects on age-0 channel catfish. *Trans. Am. Fish. Soc.*, 111: 749-754.

Thune, R. L., Stanley, L. A. and Cooper, R. K., 1993. Pathogenesis of Gram-negative bacterial infections in warmwater fish. *Annu. Rev. Fish Dis.*, 3: 37-68.

Thurston, R. V., Russo, R. C., Luedtke, R. J., Smith, C. E., Meyn, E. L., Chakoumakos, C., Wang, K. C. and Brown, C. J. D., 1984. Chronic toxicity of ammonia to rainbow trout. *Trans. Am. Fish. Soc.*, 113, 56-73.

Tiecco, G., Sebastio, C., Francioso, E., Jantillo, G. and Corbani, L., 1988. Vaccination trials against "red plague" in eels. *Dis. Aquat. Org.*, 4, 105-107.

Toranzo, A. E., Devesa, S., Romalde, J. L., Lamas, J., Riaza, A., Leiro, J. and Barja, J. L., 1995. Efficacy of intraperitoneal and immersion vaccination against *Enterococcus* sp. infection in turbot. *Aquaculture*, 134, 1-2: 17-27.

Toranzo, A. E., Novoa, B., Romalde, J. L., Núñez, S., Devesa, S., Mariño, E., Silva, R., Martinez, E., Figueras, A. and Barja, J. L., 1993. Microflora associated with diseases turbot (*Scophthalmus maximus*) from three farms in northwest Spain. *Aquaculture*, 114: 189-202.

Torkildsen, L., Samelsen, O.B., Lunestad, B. T. and Bergh Ø., 2000. Minimum inhibitory concentrations of chloramphenicol, florfenicol, trimethoprim/ sulphadiazine and flumequine in seawater of bacteria associated with scallops *Pecten maximus* larvae. *Aquaculture*, 185: 1-12.

*Trust, T. J. and Sparrow, R. A. H., 1974. The bacterial flora of alimentary tract of freshwater salmonid fishes. *Can. J. Microbiol.*, 20: 1219-1228.

*Tucker, J. W., Jr., 1993. Characteristics of some warm water marine fish with aquaculture potential. In: Proceedings of The First International

Symposium on Aquaculture Technology and Investment Opportunities. Ministry of Agriculture and Water, Riyadh, Saudi Arabia, pp. 69-88.

Udey, L. R. and Fryer, J. L., 1978. Immunisation of fish with bacterins of *Aeromonas salmonicida*. *Mar. Fish. Rev.*, 40, 3: 12-17.

Ugajin, M., 1979. Studies on the taxonomy of major microflora on the intestinal contents of salmonids. *Bull. Jpn. Soc. Sci. Fish.*, 45: 721-731.

Ullah, M. A. and Arai, T., 1983. Pathological activities of the naturally occurring strains of *Edwardsiella tarda*. *Fish Pathol.*, 18, 2: 65-70.

*Vandepitte, J., Lemmens, P. and Swart, L. De., 1983. Human edwardsiellosis traced to ornamental fish. *J. Clin. Microbiol.*, 17, 1: 165-167.

Vigneulle, M and Baudin Laurencin, F., 1995. *Serratia liquefaciens*: A case report in turbot (*Scophthalmus maximus*) cultured in floating cages in France. *Aquaculture*, 132, 1-2: 121-124.

Virgona, J. L., 1992. Environmental factors influencing the prevalence of a cutaneous ulcerative disease (red spot) in the sea mullet, *Mugil cephalus* L., in the Clarence River, New South Wales, Australia. *J. Fish Dis.*, 15, 5: 363-378.

Wakabayashi, H. and Egusa, S., 1972. Characteristics of a *Pseudomonas* and pond cultured eels (*Anguilla japonica*). *Bull. Jpn. Soc. Sci. Fish.*, 38: 577-587.

Wakabayashi, H., Egusa, S. and Fryer, J. L., 1980. Characteristics of filamentous bacteria isolated from a gill disease of salmonids. *Can. J. Fish. Aquat. Sci.*, 37, 1499-1504.

Wakabayashi, H., Hikida, M. and Masumura, K., 1986. *Flexibacter maritimus* sp. nov., a pathogen of marine fishes. *Int. J. Syst. Bacteriol.*, 36, 396-398.

Wakabayashi, H., Kanai, K., Hsu, T. C. and Egusa, S., 1981. Pathogenic activities of *Aeromonas hydrophila* biovar. *hydrophila* (Chester) Popoff and Veron, 1976 to fishes. *Fish Pathol.*, 15: 319-325.

*Wang, Z. and Zhao, S., 1995. On effect of water quality change on fish culture. Shandong Fish. Qilu Yuye, 12, 2: 36 –38.

Ward-Rainey, N., Rainey, F. A. and Stackebrandt, E., 1996. A study of the bacterial flora associated with *Holothuria atra*. *J. Exp. Mar. Biol. Ecol.*, 203, 1: 11-26.

Wedemeyer, G. A Meyer, F. P. and Smith, L., 1999. Environmental stress and fish diseases. Narendra Publishing House, Delhi. 192pp.

Westerdahl, A., Olsson, J. C. and Kjelleberg, S., 1991. Isolation and characterisation of turbot (*Scophthalmus maximus*) isolated associated bacteria with inhibitory effects against *Vibrio anguillarum*. *Appl. Environ. Microbiol.*, 57: 2223-2228.

Winton, J. R., 1998. Molecular approaches to fish vaccines. *J. Appl. Ichthyol.*, 14, 3-4: 153-158

Witters, H. E., 1998. Disorders associated with environmental pH. In: Fish diseases and disorders. Volume 2. Non infectious disorders (ed. Leatherland, J. F. and Woo, P. T. K.), CABI publishing, New York. 188-206.

Wodzinski, R. J., 1979. Introduction to extracellular enzymes: From the ribosomes to the Market place. Advances in applied microbiology (ed. Pearlman, D), Academic Press, 25, 1-6.

Wolke, R. E and Meade, T. L., 1974. Nocardiosis in Chinook salmon. *J. Wildl. Dis.*, 10: 149-154.

*Wood, C. M., 1993. Ammonia and urea metabolism and excretion. In: The Physiology of Fishes (ed. Evans, D. H.). CRC Marine Science Series. Boca Raton, Florida, pp. 379-425.

Wood, G. L. and Washington, J. A., 1995. Antibacterial susceptibility tests: dilution and disk diffusion methods. Manuel of Clinical Microbiology. 6th edition (Murray et al.) ASM Press, Washington.

*Wood, J. W. and Ordal, E. J., 1958. Tuberculosis in Pacific salmon and steelhead trout. Res. Briefs-Fish. Comm. Oregon, 25, 38pp.

Wright, M., 1991. Antibiotic and antifouling properties of marine invertebrate extracts: Comparative aspects of sponges and gorgonians. In: Bioactive compounds from marine organisms with emphasis on the Indian Ocean. An Indo United States symposium. (ed. Thompson, M. F., Sarojini, R., Nagabhushanam, R). pp. 351-356.

Yoshimizu, M. and Kimura, T., 1976. Study on the intestinal microflora of salmonids. *Fish Pathol.*, 10, 243-259.

Yoshimizu, M., Kimura, T. and Sakai, M., 1976. Studies on the intestinal microflora of salmonids. 1. The intestinal microflora of fish reared in freshwater and seawater. *Bull. Jpn. Soc. Sci. Fish.*, 42: 91-99.

*- not referred in original

APPENDICES

Appendix 1. Correlations of water quality parameters, factors of water chemistry index and death trend of ornamental fish over the period from March to September 1999

	Atmospheric Temperature	Water Temperature	Do	Salinity	pH	Nitrate	Nitrite	Ammonia	Microbial load	Factor1	Factor2	Factor3	Poma-centrids	Callyo-dontids	Siganids	Chaeto-dontids	Serranids	Acanth-urids	Others	Total
Atmospheric Temperature	1	0.618*	-0.329	-0.350	-0.402	-0.438	-0.584*	-0.020	-0.342	-0.894**	-0.468	-0.161	0.264	-0.039	0.213	0.208	-0.086	-0.061	0.147	0.142
Water Temperature	0.618*	1	-0.172	-0.185	-0.301	-0.643*	-0.362	0.378	0.206	-0.795**	-0.704*	-0.166	0.565*	0.125	-0.204	0.144	-0.102	0.060	0.295	0.218
Dissolved oxygen	-0.329	-0.172	1	0.292	0.174	0.168	0.219	-0.228	-0.014	0.261	0.533*	0.184	-0.118	0.139	0.217	-0.425	-0.210	-0.177	-0.11	-0.179
Salinity	-0.350	-0.185	0.292	1	-0.054	0.027	-0.058	-0.534*	-0.200	0.270	0.441	0.837**	-0.237	-0.129	-0.507	-0.084	0.039	-0.156	-0.214	-0.274
pH	-0.402	-0.301	0.174	-0.054	1	0.435	0.275	0.083	0.201	0.400	0.526	-0.308	-0.238	-0.331	0.309	-0.144	-0.485	-0.232	-0.388	-0.400
Nitrate	-0.438	-0.643*	0.168	0.027	0.435	1	0.532	-0.203	0.089	0.740**	0.653*	-0.299	-0.626*	-0.481	0.237	-0.133	-0.299	-0.373	-0.628*	-0.575*
Nitrite	-0.584*	-0.362	0.219	-0.058	0.275	0.532	1	-0.007	0.364	0.709**	0.293	-0.31	-0.310	0.068	0.077	-0.047	0.118	-0.044	-0.138	-0.062
Ammonia	-0.020	0.378	-0.228	-0.534*	0.083	-0.203	-0.007	1	0.789**	-0.092	-0.687**	-0.655*	0.381	0.324	-0.171	0.051	-0.213	0.067	0.187	0.153
Microbial load	-0.342	0.206	-0.015	-0.200	0.201	0.089	0.364	0.789**	1	0.273	-0.378	-0.520	0.069	0.308	-0.199	-0.039	-0.214	-0.076	-0.034	-0.034
Factor1	-0.894**	-0.795**	0.261	0.270	0.400	0.740**	0.709**	-0.092	0.273	1	0.590*	0.000	-0.508	-0.095	-0.069	-0.164	0.006	-0.118	-0.353	-0.305
Factor2	-0.468	-0.704**	0.533*	0.441	0.526	0.653*	0.293	-0.687**	-0.378	0.590	1	0.307	-0.567*	-0.408	0.314	-0.266	-0.182	-0.265	-0.481	-0.469
Factor3	-0.161	-0.166	0.184	0.837**	-0.308	-0.299	-0.310	-0.655*	-0.520	-0.0003	0.307	1	-0.049	0.020	-0.375	-0.048	0.340	0.130	0.100	0.062
Pomacentrids	0.264	0.565*	-0.118	-0.237	-0.238	-0.626*	-0.310	0.381	0.069	-0.508	-0.567*	-0.049	1	0.076	-0.186	0.136	0.093	0.598*	0.740**	0.582*
Callyodontids	-0.039	0.125	0.139	-0.129	-0.331	-0.481	0.067	0.324	0.308	-0.095	-0.408	0.020	0.075	1	-0.023	0.222	0.443	0.238	0.535*	0.638*
Siganids	0.213	-0.204	0.217	-0.507	0.309	0.237	0.077	-0.171	-0.199	-0.069	0.314	-0.375	-0.186	-0.023	1	-0.222	-0.219	-0.156	-0.148	-0.054
Chaetodontids	0.208	0.144	-0.425	-0.084	-0.144	-0.133	-0.047	0.051	-0.039	-0.164	-0.266	-0.048	0.136	0.222	-0.222	1	0.077	0.031	0.378	0.429
Serranids	-0.086	-0.102	-0.210	0.039	-0.485	-0.299	0.118	-0.213	-0.214	0.006	-0.182	0.340	0.093	0.443	-0.219	0.077	1	0.757**	0.609*	0.701*
Acanthurids	-0.061	0.060	-0.177	-0.156	-0.232	-0.373	-0.044	0.067	-0.076	-0.118	-0.265	0.130	0.598*	0.238	-0.156	0.031	0.757**	1	0.831**	0.800*
Others	0.147	0.295	-0.110	-0.214	-0.388	-0.628*	-0.138	0.187	-0.034	-0.353	-0.481	0.100	0.741**	0.535*	-0.148	0.378	0.609*	0.831**	1	0.968*
Total	0.142	0.218	-0.179	-0.274	-0.400	-0.575	-0.062	0.153	-0.034	-0.305	-0.469	0.062	0.582*	0.638*	-0.054	0.429	0.701**	0.800*	0.968**	1

* - Correlation is significant at the 0.05 level; ** - Correlation is significant at the 0.01 level.

Appendix 2. Results of antibiotic sensitivity studies: s- sensitive; i-intermediate sensitivity; r- resistant

Name of isolate	P	A	Cx	N	K	G	E	S	C	R	Na	Ap
Alcaligenes	S(15)	R	R	S (23)	S (24)	S (22)	R	S (24)	S (23)	S (18)	S (26)	R
V. furnissi	R	I (18)	I (11)	I (14)	S (20)	S (23)	R	S (25)	S (24)	S (22)	S (25)	R
Pseudomonas	R	S (23)	S (26)	S (20)	S (23.5)	S (24.5)	S (24)	S (22)	S (21)	S (26)	S (25)	S(14)
Flavobacterium	I (26)	S (28)	S (21)	I (18.5)	S (24)	S (27.5)	S (29.5)	S	S (30)	S (22)	S (2(25)	R
Alcaligenes	I (23)	R	R	S (20)	R	S (22)	R	S (16)	S (22)	I (17)	R	S (12)
Enterobacteriaceae	R	S (22.5)	S (32)	I (17)	S (20)	S (27)	S (22.5)	S (24)	S (30)	S (37)	R	S (18)
Acinetobacter	R	S (26)	I (12.5)	I (18)	S (24)	S (25)	S (24)	S (22)	S (20)	S(20)	S (25)	R
Pseudomonas	R	R	I (12)	S (21)	R	S (27)	S (23)	S (30)	S (27)	I (15)	S (27)	R
Acinetobacter	R	S (26)	S (31)	S (23.5)	S (23.5)	S (30)	S (30)	S (25)	S (20)	S (30)	S (28)	R
Acinetobacter	R	S (34)	S (14)	S (24.5)	S (32)	S (25.5)	S (27.5)	S (29)	S (32)	I (17)	S (32)	R
Pseudomonas	I (22)	S (21)	I (12)	S (23)	S (24)	S (27)	S (28)	S (34)	S (30)	S (22)	S (25)	R
Flavobacterium	S (26)	S (37.5)	S (31)	S (20)	S (24)	S (28.5)	S (26)	S (24)	S (20)	S (26)	S (25)	R
V. mediterranei	R	R	S (42)	S (26)	S (25)	S (20)	S (32)	S (24)	S (32)	S (36)	S (30)	R
Pseudomonas	R	S (29)	S (32)	S (24)	S (26)	S (27)	I (16)	S (24)	R	S (32)	S (19)	R
V. mediterranei	R	S (26)	S (36)	S (22)	S (26)	S (30)	S (22.5)	R	R	S (27)	S (29)	S (13)
V. mediterranei	R	S (30)	S (34)	S (22)	S (24)	S (26)	I (21)	S (26)	I (13)	S (20)	S (25)	R
Enterobacteriaceae	R	R	R	I (16)	S (19)	S (21)	R	S (16)	S (18)	I (17)	R	R
S. marcescens	R	R	R	I (16)	I (16)	S (23.5)	I (20)	S (17)	S (20)	R	S (22)	R
Flavobacterium	S (18)	S (33)	S (13.5)	S (20)	S (24)	S (25)	I (19)	S (22)	R	S (28)	S (20)	R
V. mediterranei	R	S (26.5)	S (38)	S (20)	S (22.5)	S (24)	I (22)	S (26)	S (18)	S (25)	S (20)	R
Flavobacterium	S (25)	S (29.5)	S (40)	I(18)	S (22)	S (26)	S (23.5)	S (33)	I (17)	S (28)	S (23)	R
V. furnissi	R	S (24)	S (32)	I (18)	S (21)	S (25)	S (23.5)	S (24)	R	R	S (20)	R
Acinetobacter	R	S (28)	S (45)	I (18)	S (22.5)	S (27)	I (17)	S (26)	R	S (24)	S (22)	R
Serratia	R	S (28.5)	S (34)	I (19)	S (23)	S (25)	S (27.5)	S (22)	S (22)	S (28)	S (20)	R
Alcaligenes	S (35)	S (29)	S (40)	S (20.5)	S (26)	S (26.5)	I (21)	S (23)	S (18)	S (32)	S (25)	R
Flavobacterium	R	R	S (19)	S (20)	S (26)	S (21.5)	S (21.5)	S (37)	S (19)	S (21)	S (22)	R
Flavobacterium	R	R	R	R	R	R	R	S (16)	S (30)	S (30)	S (24)	R
Flavobacterium	R	R	S (19)	R	R	S (24)	S (22.5)	S (25)	S (30)	S (23)	S (34)	R
Alcaligenes	R	R	S (14)	R	R	S (23)	I (19)	S (26)	S (30)	S (24)	S (31)	R
Flavobacterium	R	R	R	R	S(20)	S(20)	I(18)	S(22)	S(35)	S(38)	S(29)	R
Flavobacterium	R	R	R	I (19)	S (18)	S (19)	R	R	R	S (18)	S (24)	R

Name of isolate	Ba	Do	Co	Mt	Sm	Sz	Tm	Cf	Fz	Nr	Nf	T	O
Alcaligenes	R	S (20)	S (23)	R	S (26)	I (14)	S (34)	R	S (20)	R	S (17)	S (20)	S (26)
V. furnissi	R	S (18)	S (22)	R	R	S (28)	S (25)	R	I (14)	R	I (15)	R	S (23)
Pseudomonas	R	S (36)	S (42)	R	S (26)	S (25)	S (37)	S (34)	S (20)	S (21)	S (20)	S (27)	S (22)
Flavobacterium	R	S (31)	S (17)	R	S (23)	S	S (33)	S	S	S	S (24)	S (31)	S (31)
Alcaligenes	R	S (22)	R	R	R	S (18)	R	S (34)	R	R	R	S (21)	R
Enterobacteriaceae	S (15)	S (32)	S (29)	I (13)	R	R	S (20)	S (30)	I (16)	S (23)	S (18)	S (22)	S (20)
Acinetobacter	R	R	S	R	S (23)	R	S (31)	S (30)	R	R	S (17)	S (30)	S (30)
Pseudomonas	R	S (18)	R	R	R	R	R	S (36)	R	R	S(17)	S (18)	S (31)
Acinetobacter	S (16)	S (35)	S (17)	I (12)	S (20)	S (24)	R	S (19)	R	R	S (17)	S (30)	S (27)
Acinetobacter	R	S (36)	S (44)	R	R	R	R	S (40)	I (16)	S (24)	S (25)	S (28)	S (30)
Pseudomonas	I (14)	S (32)	S (44)	R	S (25)	I (14)	R	S (48)	S (30)	S (30)	S (18)	S (26)	S (30)
Flavobacterium	I (14)	S (34)	S (42)	R	S (23)	S (22)	R	S (30)	S (17)	S (23)	S (20)	S (27)	S (30)
V. mediterranei	S (17)	S (38)	S (35)	R	R	S (22)	R	S (26)	S (27)	S (25)	S (28)	S (33)	S (25)
Pseudomonas	I (12)	S (39)	S (40)	R	S (21)	S (24)	S (22)	S (36)	R	R	S (22)	S (24)	S (32)
V. mediterranei	S (15)	S (34)	S (34)	R	S (20)	R	R	S (28)	R	S (17)	S (18)	S (33)	S (18)
V. mediterranei	R	S (25)	S (47)	R	S (26)	S (26)	S (31)	S (44)	S (24)	S (18)	I (15)	S (38)	S (24)
Enterobacteriaceae	R	R	R	R	S (27)	R	R	S (30)	R	R	S (17)	S (19)	I (15)
S. marcescens	R	R	S (24)	R	R	I (16)	S (24)	S (32)	R	R	S (17)	S (23)	R
Flavobacterium	R	R	S (23)	R	S (18)	R	S (44)	S (28)	S (19)	S (18)	R	S (37)	S (33)
V. mediterranei	R	S (21)	R	R	R	S (30)	S (38)	S (32)	S (20)	S (20)	I (16)	S (36)	S (24)
Flavobacterium	R	R	S (23)	R	S (18)	R	S (44)	S (28)	S (19)	S (18)	R	S (37)	S (33)
V. furnissi	R	I (13)	R	R	S (20)	S (20)	S (43)	S (30)	R	S (18)	I (15)	S (28)	S (30)
Acinetobacter	R	S (29)	S (42)	R	S (26)	S (36)	S (45)	S (38)	S (18)	R	I (15)	S (35)	S (24)
Serratia	R	S (32)	S (50)	R	S (24)	R	S (41)	S (28)	S (18)	S (18)	S (19)	S (30)	S (24)
Alcaligenes	R	S (34)	S (16)	R	S (25)	S (25)	S (40)	S (38)	R	R	S (19)	S (34)	R
Flavobacterium	I	S (23)	S (20)	R	R	R	S (35)	S (33)	S (22)	S (20)	R	S (25)	I (17)
Flavobacterium	I	S (32)	S (20)	R	S (20)	R	S (31)	R	R	R	R	S (24)	S (20)
Flavobacterium	R	S (19)	S (29)	R	R	R	S (16)	S (39)	S (18)	S (23)	I (14)	I (18)	I (18)
Alcaligenes	R	I (15)	S (28)	R	R	R	R	S (34)	S (20)	R	I (15)	S (33)	S (22)
Flavobacterium	R	S (36)	R	R	S (33)	S (36)	S (27)	S (41)	S (25)	S (30)	S (20)	S (22)	S (25)
Flavobacterium	R	S (23)	S (28)	R	S (26)	R	R	S (29)	R	R	S (20)	S (24)	S (19)